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(54) Title: NON-TRAUMATIC ADMINISTRATION OF GENE DELIVERY VEHICLES

(57) Abstract

The non-traumatic administration of one or more gene delivery vehicles ("GDVs") to an animal. Such non-traumatic administration provides significant advantages over prior methods of administration because it does not require any injection or other invasion of the recipient, and therefore is much less injurious to the recipient. Further, because there is no injection or invasion, there is typically less need for highly skilled personnel to administer the GDVs, there is less need for sterile conditions, and there is less risk of infection, injury and other side effects.

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NON-TRAUMATIC ADMINISTRATION OF GENE DELIVERY VEHICLES

Field of the Invention

The field of the present invention is the administration of recombinant nucleic acid vectors and other vehicles containing nucleic acid sequences having desirable properties, such as the expression of a desired substance or the ability to incorporate into a specified nucleic acid sequence, into an animal.

Background of the Invention

Recent advances in the field of biotechnology, including the engineering of desirable nucleic acid molecules, have given rise to significant advances in the treatment of diseases such as cancer, genetic diseases, arthritis and AIDS. Many of these advances involve the administration of desirable nucleic acid molecules to a subject, particularly human subjects. Non-traumatic (non-parenteral) administration, such as oral administration, of such nucleic acid molecules can provide simpler, easier, less injurious administration of the molecules, and can also reduce the need for sterile procedures.

However, despite the advantages of non-traumatic administration, desirable nucleic acids suitable for the treatment of such diseases or other pathogenic states have not been administered via non-traumatic routes because the nucleic acids have not been obtained in an isolated, purified, dry form suitable for combination with the necessary pharmaceutically acceptable carriers or diluents, yet capable of withstanding the rigors, such as extreme pH, of passage through the mouth, stomach and intestines. For example, the physical preparation of a delivery system such as tablets and capsules traditionally requires the active ingredients and excipients be in an anhydrous form. Residual water will attack the dosage preparation, resulting in an unstable product. Further, in addition to the delivery system, the nucleic acid molecules and product as a whole must be stable in a dried state. This is of particular concern for nucleic acid molecules, because, when such molecules are stored in an aqueous environment, the molecules are typically only stable at reduced temperatures.

Thus, there is a need for compositions comprising desirable nucleic acid molecules suitable for non-traumatic administration, and for methods of non-traumaticly administering such molecules.

The present invention provides such methods of non-traumaticly administering nucleic acid molecules, compositions containing such nucleic acid molecules

that are adequately isolated and purified to permit convenient production of tablets, and other, related advantages.

Summary of the Invention

In one aspect, the present invention is directed to a method of introducing a nucleic acid molecule to an animal or patient . comprising non-traumaticly administering a composition comprising a gene delivery vehicle to the animal, the gene delivery vehicle directing the expression of at least one substance in a host cell containing the gene delivery vehicle, the substance not naturally expressed by the gene delivery vehicle, in combination with a pharmaceutically acceptable carrier or diluent.

In another aspect, the present invention is directed towards a method of introducing a nucleic acid molecule to an animal or patient comprising non-traumaticly administering a composition comprising a gene delivery vehicle to the animal, the gene delivery vehicle containing at least one biologically active nucleic acid sequence wherein such biological activity is not naturally contained within the gene delivery vehicle, in combination with a pharmaceutically acceptable carrier or diluent.

In a further aspect, the present invention is directed towards a method of introducing a nucleic acid molecule to an animal or patient comprising non-traumaticly administering a composition comprising a gene delivery vehicle to the animal, the gene delivery vehicle containing a nucleic acid sequence that is not naturally contained within the gene delivery vehicle, in combination with a pharmaceutically acceptable carrier or diluent.

In preferred embodiments, the present invention includes methods as set forth above wherein the pharmaceutically acceptable carrier or diluent enhances the non-traumatic administration of the gene delivery vehicle. In other preferred embodiments, in the methods set forth above, the substance or the biological activity is not exhibited in the host cell containing the GDV prior to the non-traumatic administration. In further embodiments, the biological activity is not exhibited within the animal prior to non-traumatic administration. In alternative embodiments, the biological activity is reduced or sub-optimal within the animal prior to non-traumatic administration.

In other preferred embodiments, in the method set forth above, the biological activity complements, activates, replaces and/or suppresses a biological activity present in a host cell within the animal prior to non-traumatic administration.

In a further aspect, the present invention provides a composition suitable for non-traumatic administration of a nucleic acid molecule to an animal, the composition comprising an anhydrous, lyophilized gene delivery vehicle that directs the expression of at least one substance, or encodes a biologically active nucleic acid molecule, in a host cell containing the gene delivery vehicle, the substance or biologically active nucleic acid

molecule not naturally expressed by or contained within the gene delivery vehicle, in combination with a pharmaceutically acceptable carrier or diluent.

These and other aspects of the present invention will become evident upon reference to the following detailed description. In addition, various references are set forth herein which describe in more detail certain procedures or compositions; such references are incorporated by reference in their entirety.

Brief Description of the Drawings

Figure 1 is a schematic illustration of p31N2R5(+).

Figure 2 is a schematic illustration of pN2R3(-).

Figure 3 is a schematic illustration of p31N25Δ(+).

Figure 4 is a schematic illustration of pN2R3(+).

Figure 5 is a schematic illustration of pN2R5(-).

Figure 6 is a schematic illustration of p31N25Δ(+).

Figure 7 is a schematic illustration of pTKΔA.

Figure 8 is a schematic illustration of pPrTKΔA.

Figure 9 is a schematic illustration of pTK-1 and pTK-3.

Figure 10 is a bar graph which illustrates the effect of Ganciclovir on CT26, CT26 β-gal and CT26TK Neo cells.

Figure 11 is a graph which illustrates the effect of tumor volume over time in a Ganciclovir dose study of mice injected with CT26TK Neo.

Figure 12 is a series of four photographs of mice, illustrating the effect of different dose regimens of Ganciclovir on intraperitoneal tumor growth.

Figure 13 is a series of four photographs of mice, illustrating the effect of different dose regimens of Ganciclovir on subcutaneous tumor growth.

Figure 14 is a graph illustrating the effect of Ganciclovir in CT26 versus CT26TK Neo cells.

Figure 15 is a graph demonstrating retention of viral activity upon reconstitution of a representative recombinant retrovirus lyophilized in a formulation buffer containing mannitol.

Figure 16 is a graph demonstrating retention of viral activity upon reconstitution of a representative recombinant retrovirus lyophilized in a formulation buffer containing lactose.

Figure 17 is a graph demonstrating retention of viral activity upon reconstitution of a representative recombinant retrovirus lyophilized in a formulation buffer containing trehalose.

Figures 18A-18D are representative graphs comparing stability of liquid non-lyophilized recombinant retrovirus stored at -80°C versus lyophilized formulated recombinant

retrovirus stored at -20°C, using various saccharides. For ease of comparison, the titers have been normalized.

Detailed Description of the Invention

The present invention is directed towards the non-traumatic administration of one or more gene delivery vehicles ("GDVs") to an animal. Such non-traumatic administration provides significant advantages over prior methods of administration because it does not require any injection or other invasion of the recipient, and therefore is much less injurious to the recipient. Further, because there is no injection or invasion, there is typically less need for highly skilled personnel to administer the GDVs, there is less need for sterile conditions, and there is with less risk of infection, injury and other side effects.

I. Gene Delivery Vehicles

A "gene delivery vehicle" is a recombinant vehicle, such as a viral vector, a nucleic acid vector (such as plasmid), a naked nucleic acid molecule such as genes, a nucleic acid molecule complexed to a polycationic molecule capable of neutralizing the negative charge on the nucleic acid molecule and condensing the nucleic acid molecule into a compact molecule, a bacterium, and certain eukaryotic cells such as a producer cell, that are capable of delivering a nucleic acid molecule having one or more desirable properties to host cells in an organism. As discussed further below, the desirable properties include the ability to express a desired substance, such as a protein, enzyme or antibody, and/or the ability to provide a biological activity, which is where the nucleic acid molecule carried by the GDV is itself the active agent without requiring the expression of a desired substance. One example of such biological activity is gene therapy where the delivered nucleic acid molecule incorporates into a specified gene so as to inactivate the gene and "turn off" the product the gene was making.

Typically, the GDV is an assembly that carries a nucleic acid molecule (or sequence), such molecule often capable of expressing sequences or genes of interest. In the context of protein expression, the GDV must include promoter elements such as for RNA Polymerase II or RNA replicase, and may include a signal that directs polyadenylation. In addition, the GDV preferably includes a molecule that, when transcribed, is operably linked to the molecules or genes of interest and acts as a translation initiation sequence. The GDV may include a selectable marker such as neomycin, thymidine kinase, hygromycin, phleomycin, histidinol, or dihydrofolate reductase (DHFR), as well as one or more restriction sites and a translation termination sequence. In addition, if the GDV is used to make a retroviral particle, the GDV must include a retroviral packaging signal and LTRs appropriate to the retrovirus used, provided these are not already present. The GDV can also be used in combination with other viral vectors or inserted physically into cells or tissues as described

below. The GDV may include a sequence that encodes a protein or active portion of the protein, antisense or ribozyme. Such sequences may be designed to inhibit MHC antigen presentation in order to suppress the immune response of cytotoxic T-lymphocytes against a transplanted tissue.

Particularly preferred viral vectors for use as a GDV within the present invention include recombinant retroviral vectors and recombinant adenovirus vectors. The construction of recombinant retroviral vectors is described in greater detail in an application entitled "Recombinant Retroviruses" (this reference and all other references cited with this application are expressly incorporated herein in their entirety). These recombinant retroviral vectors may be used to generate transduction competent retroviral vector particles by introducing them into appropriate packaging cell lines. Similarly, adenovirus vectors may also be readily prepared and utilized given the disclosure provided herein (see also Berkner, *Biotechniques 6*:616-627, 1988, and Rosenfeld et al., *Science 252*:431-434, 1991, WO 93/07283, WO 93/06223, and WO 93/07282).

In another preferred embodiment, the GDV is a Sindbis RNA expression vector that includes, in order, a 5' sequence which is capable of initiating transcription of a Sindbis virus, a nucleotide sequence encoding Sindbis non-structural proteins, a viral junction region, a heterologous sequence, a Sindbis RNA polymerase recognition sequence, and a stretch of 25 consecutive polyadenylate residues. A wide variety of heterologous sequences may be included in the GDV. Within various embodiments of the invention, the GDV may contain (and express, within certain embodiments) two or more heterologous sequences.

Other viral vectors suitable for use in the present invention include, for example, poliovirus (Evans et al., *Nature 339*:385-388, 1989, and Sabin, *J. of Biol. Standardization 1*:115-118, 1973); rhinovirus (Arnold, *J. Cell. Biochem.* L401-405, 1990); pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., *PNAS 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112 and 4,769,330; WO 89/01973); SV40 (Mulligan et al., *Nature 277*:108-114, 1979); influenza virus (Luytjes et al., *Cell 59*:1107-1113, 1989; McMicheal et al., *The New England Journal of Medicine 309*:13-17, 1983; and Yap et al., *Nature 273*:238-239, 1978); pavovirus such as adeno-associated virus (Samulski et al., *Journal of Virology 63*:3822-3828, 1989, and Mendelson et al., *Virology 166*:154-165, 1988); herpes (Kit, *Adv. Exp. Med. Biol. 215*:219-236, 1989); HIV; measles (EP 0 440,219); measles (EP 0 440,219); astrovirus (Munroe, S.S. et al., *J. Vir. 67*:3611-3614, 1993); Semliki Forest Virus, and coronavirus, as well as other viral systems (*e.g.*, EP 0,440,219; WO 92/06693; U.S. Patent No. 5,166,057). In addition, viral carriers may be homologous, non-pathogenic (defective), replication competent virus (*e.g.*, Overbaugh et al., *Science 239*:906-910, 1988).

In a preferred embodiment, where the GDV is a retroviral vector, the nucleic acid molecules carried by the retroviral vector should be of a size sufficient to allow production of viable virus. Within the context of the present invention, the production of any measurable titer of infectious virus on susceptible monolayers is considered to be "production of viable virus." Within preferred embodiments, a heterologous sequence within the retroviral vector GDV will comprise at least 100 bases, at least 2 kb, 3.5 kb, 5 kb, or 7 kb, or even a heterologous sequence of at least 8 kb.

A nucleic acid molecule without any covering, such as a viral capsid or bacterial cell membrane, is also suitable for use as a GDV within the present invention. Such "naked" nucleic acids include plasmids, viral vectors without coverings, and even naked genes without any control region. The GDV may be either DNA or RNA, or may be a combination of the two, comprising both DNA and RNA in a single molecule.

In another alternative embodiment, the GDV is a liposome. Liposomes are small, lipid vesicles comprised of an aqueous compartment enclosed by a lipid bilayer, typically spherical or slightly elongated structures and several hundred angstroms in diameter. Liposomes offer several readily exploited features. Under appropriate conditions, the liposome can fuse with the plasma membrane of a target cell or with the membrane of an endocytic vesicle within a cell which has internalized the liposome, thereby disgorging its contents into the cytoplasm. Prior to interaction with the surface of a target cell, however, the liposome membrane acts as a relatively impermeable barrier which sequesters and protects its contents, for example from degradative enzymes in the plasma. Liposomes have for this reason also been referred to as "micropills". Additionally, because a liposome is a synthetic structure, custom-formulated liposomes can be designed that incorporate desirable features. (Stryer, L., *Biochemistry*, pp236-240 1975 (W.H. Freeman, San Francisco); Szoka et al., *Biochim. Biophys. Acta 600*:1-18 (1980); Bayer et al., *Biochim. Biophys. Acta. 550*:464 (1979); Rivnay et al., *Meth. Enzymol. 149*:119 (1987); Wang et al., *PNAS 84*: 7851, 1987 and, Plant et al., *Anal. Biochem. 176*:420 (1989).

A bacterial cell suitable for use as a GDV within the present invention includes a bacterium that expresses a cytotoxic agent, such as an anti-tumor agent, on its cell surface or exported from the bacterium. Representative examples include BCG (Stover, *Nature 351*:456-458, 1991) and Salmonella (Newton et al., *Science 244*:70-72, 1989). Eukaryotic cells suitable for use in the present invention include producer cells and *ex vivo* transduced cells.

Within one embodiment of the present invention, the GDV comprises a nucleic acid molecule under the transcriptional control of an event-specific promoter, such that upon activation of the event-specific promoter the nucleic acid molecule is expressed. Numerous event-specific promoters may be utilized within the context of the present invention, including for example, promoters which are activated by cellular proliferation (or

are otherwise cell-cycle dependent) such as the thymidine kinase or thymidilate synthase promoters (Merrill, *Proc. Natl. Acad. Sci. USA 86*:4987-91, 1989; Deng et al., *Mol. Cell. Biol. 9*:4079-82, 1989); promoters such as the α- or β- interferon promoters which are activated when a cell is infected by a virus (Fan and Maniatis, *EMBO J. 8*(1):101-110, 1989; Goodbourn et al. *Cell 45*:601-610, 1986); and promoters which are activated by the presence of hormones (*e.g.*, estrogen response promoters; *see* Toohey et al., *Mol. Cell. Biol. 6*:4526-38, 1986).

Within a preferred embodiment, a recombinant viral vector (preferably, but not necessarily, a recombinant MLV retrovirus) carries a gene expressed from an event-specific promoter, such as a cell cycle-dependent promoter (*e.g.*, human cellular thymidine kinase or transferrin receptor promoters), which will be transcriptionally active primarily in proliferating cells, such as tumors. In this manner, replicating cells which contain factors capable of activating transcription from these promoters are preferentially affected (*e.g.*, destroyed) by the agent produced by the GDV.

Within another embodiment of the present invention, the GDV comprises a nucleic acid molecule under the transcriptional control of a tissue-specific promoter, such that upon activation of the tissue-specific promoter the nucleic acid molecule is expressed. A wide variety of tissue-specific promoters may be utilized within the context of the present invention. Representative examples of such promoters include: liver-specific promoters such as Phospho-Enol-Pyruvate Carboxy-Kinase (Hatzogiou et al., *J. Biol. Chem. 263:* 17798-808, 1988; Benvenisty et al., *Proc. Natl. Acad. Sci. USA 86*:1118-22, 1989; Vaulont et al., *Mol. Cell. Biol. 9*:4409-15, 1989), the albumin promoter and the alpha-fetoprotein (AFP) promoter (Feuerman et al., *Mol. Cell. Biol. 9*:4204-12, 1989; Camper and Tilghman, *Genes Develop. 3*:537-46, 1989); B cell specific promoters such as the IgG promoter; breast carcinoma or hepatocellular carcinoma specific promoters such as carcinoembryonic antigen (CEA) promoter (Schrewe et al., *Mol. and Cell. Biol. 10*:2738, 1990); pancreatic acinar cell specific promoters such as the elastase promoter (Swift et al., *Genes Develop. 3*:687-96, 1989); breast epithelial specific promoters such as the casein promoter (Doppler et al., *Proc. Natl. Acad. Sci. USA 86*:104-08, 1989); erythroid specific-transcription promoters which are active in erythroid cells, such as the porphobilinogen deaminase promoter (Mignotte et al., *Proc. Natl. Acad. Sci. USA 86*:6458-52, 1990); α- or β- globin specific promoters (van Assendelft et al., *Cell 56*:969-77, 1989, Forrester et al., *Proc. Natl. Acad. Sci. USA 86*:5439-43, 1989); promoters which regulate skeletal muscle such as the myo-D binding site (Burden, *Nature 341:*716, 1989; Weintraub et al., *Proc. Natl. Acad. Sci. USA 86*:5434-38, 1989); promoters which are specific for β cells of the pancreas, such as the insulin promoter (Ohlsson et al., *Proc. Natl. Acad. Sci. USA 85*:4228-31, 1988; Karlsson et al., *Mol. Cell. Biol. 9:*823-27, 1989); promoters that are specific for the pituitary gland, such as the growth hormone factor promoter (Ingraham et al., *Cell 55:*519-29, 1988; Bodner et al., *Cell 55*:505-

18, 1988); promoters which are specific for melanocytes, such as the tyrosine hydroxylase promoter; breast carcinoma specific promoters such as the HER2/neu promoter (Tal et al., *Mol. and Cell. Biol. 7*:2597, 1987); liver-specific promoters such as the alcohol dehydrogenase (ADH) promoter (Felder, *Proc. Natl. Acad. Sci. USA 86*:5903-07, 1989); T-cell specific promoters such as the T-cell receptor promoter (Anderson et al., *Proc. Natl. Acad. Sci. USA 85*:3551-54, 1988; Winoto and Baltimore, *EMBO J. 8*:729-33, 1989); osteoblast or bone-specific promoters such as the osteocalcin promoter (Markose et al., *Proc. Natl. Acad. Sci. USA 87*:1701-1705, 1990; McDonnell et al., *Mol. Cell. Biol. 9*:3517-23, 1989; Kerner et al., *Proc. Natl. Acad. Sci. USA 86*:4455-59, 1989) the IL-2 promoter, IL-2 receptor promoter, the whey (WAP) promoter, and the MHC Class II promoter.

A variety of other elements which control gene expression may also be utilized within the context of the present invention, including for example locus-defining elements such as the β-globin gene and the T cell marker CD2. In addition, elements which control expression at the level of splicing and nuclear export are the β-globin intron sequences, the rev and rre elements in HIV-1, and the CTE element in the D-type masonpfizer monkey retrovirus.

Within preferred embodiments of the invention, the GDV is a retroviral vector and the gene produces an agent against a tumor, the gene being under control of a tissue-specific promoter having specificity for the tissue of tumor origin. Since the retroviral vector preferentially integrates into the genome of replicating cells (for example, normal liver cells are only slowly replicating, while those of a hepatocarcinoma are replicating more quickly), these two levels of specificity (viral integration/replication and tissue-specific transcriptional regulation) lead to preferential killing of tumor cells.

Within yet another related embodiment of the present invention, the GDV comprises a nucleic acid molecule under the transcriptional control of both an event-specific promoter and a tissue-specific promoter, such that the nucleic acid molecule is maximally expressed only upon activation of both the event-specific promoter and the tissue-specific promoter. In particular, by utilizing such vectors, the substance expressed from the nucleic acid molecule is expressed only in cell types satisfying both criteria (*e.g.*, in the example above, combined promoter elements are functional only in rapidly dividing liver cells). Within preferred embodiments of the invention, the number of transcriptional promoter elements may also be increased, in order to improve the stringency of cell-type specificity.

Transcriptional promoter/enhancer elements as discussed above need not necessarily be present as an internal promoter (lying between the viral LTRs for retroviruses, for example), but may be added to or replace the transcriptional control elements in the viral LTRs which are themselves transcriptional promoters, such that condition-specific (*i.e.*, event or tissue specific) transcriptional expression will occur directly from the modified viral LTR. In this case, either the condition for maximal expression will need to be mimicked in

retroviral packaging cell lines (*e.g.*, by altering growth conditions, supplying necessary transregulators of expression or using the appropriate cell line as a parent for a packaging line), or the LTR modification is limited to the 3' LTR U3 region, to obtain maximal recombinant viral titers. In the latter case, after one round of infection/integration, the 3' LTR U3 is now also the 5' LTR U3, giving the desired tissue-specific expression. Similarly, for other viral vectors, the promoters may be exogenous, or hybrids with normal viral promoter elements.

The present invention also provides eukaryotic layered vector initiation systems, which are comprised of a 5' promoter, a construct which is capable of expressing one or more heterologous nucleotide sequences, and, of replication in a cell either autonomously or in response to one or more factors, a polyadenylation sequence, and a transcription termination sequence. Briefly, eukaryotic layered vector initiation systems provide a two stage or "layered" mechanism which controls expression of heterologous nucleotide sequences. The first layer initiates transcription of the second layer, and comprises a 5' promoter, polyadenylation site, and transcription termination site, as well as one or more splice sites if desired. Representative examples of promoters suitable for use in this regard include any viral or cellular promoters such as CMV, retroviral LTRs, SV40, β-actin, immunoglobulin promoters, and inducible promoters such as the metallothionein promoter and glucocorticoid promoter. The second layer comprises a construct which is capable of expressing one or more heterologous nucleotide sequences, and, of replication in a cell either autonomously or in response to one or more factors. Within one embodiment of the invention the construct may be a Sindbis GDV as described above.

In a preferred embodiment, the present invention provides a GDV comprising a retroviral vector. The retroviral vector is preferably selected from the group comprising KT-1, KT-3, crossless backbone vectors, or a vector that employs a promoter that facilitates tissue- or event-specific expression. Further, the retroviral vector preferably includes one or both of a marker gene, such as neomycin resistance, and a "suicide gene," such as the herpes simplex virus thymidine kinase (HSVTK) gene.

The GDV is then introduced into suitable packaging cell lines, which cell lines can be selected for particularly desirable characteristics, such as where the GDVs each display amphotropic, xenotropic or polytropic characteristics. Other suitable packaging cell lines include the 293 2-3 VSV-G system, and cell lines that exhibit vector structural protein modified to facilitate targeting of the transduction of the vector to a preferred location (*e.g.*, a regional lymph node or a cell that presents a particular antigen). The cell lines can then be tested to confirm that they contain the desirable components.

Next, cell cultures are prepared, and supernatant fluids that contain the retroviral vectors are harvested. The fluids can be tested for GDV potency, typically measured in colony forming units (CFU) or plaque forming units (PFU), as appropriate. In

one approach, the GDV themselves are not further processed prior to administration to the host animal or plant. In a preferred approach, the GDV is then concentrated, purified and formulated before administration.

II. Non-traumatic Administration

Non-traumatic administration includes administration of the GDV of the present invention pursuant to traditional non-traumatic routes, such as buccal/sublingual, rectal, oral, nasal, topical, (such as transdermal and ophthalmic), vaginal, and pulmonary administration. Non-traumatic administration also includes delivery into bladder through the urethra. Thus, the GDV is not administered via an invasive route that requires entry into the living body, as by incision, injection, or insertion of an instrument into a non-natural opening. Examples of such invasive routes of administration include intraarterial, intramuscular, intraperitoneal, subcutaneous, intraocular, and intravenous administration.

Considerations for administering the compositions of the present invention include the following:

Oral administration is easy and convenient, economical (no sterility required), safe (over dosage can be treated in most cases), and permits controlled release of the active ingredient of the composition (the GDV). Conversely, there may be local irritation such as nausea, vomiting or diarrhea, erratic absorption for poorly soluble drugs, and the GDV will be subject to "first pass effect" by hepatic metabolism and gastric acid and enzymatic degradation. Further, there can be slow onset of action, efficient plasma levels may not be reached, a patient's cooperation is required, and food can affect absorption. Preferred embodiments of the present invention include the oral administration of GDV comprising retroviral vectors that contain genes encoding erythropoietin, insulin, GM-CSF, or other cytokines, or various polypeptides or peptide hormones, their agonists or antagonists, where these hormones can be derived from tissues such as the pituitary, hypothalamus, kidney, endothelial cells, liver, pancreas, bone, hemopoetic marrow, and adrenal. These polypeptides can be used for induction of growth, regression of tissue, suppression of immune responses, apoptosis, gene expression, blocking receptor-ligand interaction, immune responses and can be treatment for certain anemias, diabetes, infections, high blood pressure, abnormal blood chemistry or chemistries (*e.g.*, elevated blood cholesterol, deficiency of blood clotting factors, elevated LDL with lowered HDL). Such polypeptides can also be used to control levels of Alzheimer associated amyloid protein, bone erosion/calcium deposition, and to control levels of various metabolites such as steroid hormones, purines, and pyrimidines. Preferably, the retroviral vectors are first lyophilized, then filled into capsules and administered.

Buccal/sublingual administration is a convenient method of administration that provides rapid onset of action of the active component(s) of the composition, and avoids

first pass metabolism. Thus, there is no gastric acid or enzymatic degradation, and the absorption of GDV exhibiting peptides is feasible. There is high bioavailability, and virtually immediate cessation of treatment is possible. Conversely, such administration is limited to relatively low dosages (typically about 10-15 mg, a problem that is overcome by the present invention), and there can be no simultaneous eating, drinking or swallowing. Preferred embodiments of the present invention include the buccal/sublingual administration of GDV comprising retroviral vectors that contain genes encoding self and/or foreign MHC, or immune modulators, for the treatment of oral cancer; the treatment of Sjogren's syndrome via the buccal/sublingual administration of such retroviral vectors that contain IgA or IgE antisense genes; the treatment of gingivitis and periodontitis via the buccal/sublingual administration of IgG or cytokine antisense genes; and, many of the applications discussed above with respect to oral delivery.

Rectal administration provides a negligible first pass metabolism effect (there is a good blood/lymph vessel supply, and absorbed materials drain directly into the inferior vena cava), and the method is suitable of children, patients with emesis, and the unconscious. The method avoids gastric acid and enzymatic degradation, and the ionization of a composition will not change because the rectal fluid has no buffer capacity (pH 6.8; charged compositions absorb best). Conversely, there may be slow, poor or erratic absorption, irritation, degradation by bacterial flora, and there is a small absorption surface (about $0.05m^2$). Further, lipidophilic and water soluble compounds are preferred for absorption by the rectal mucosa, and absorption enhancers (*e.g.*, salts, EDTA, NSAID) may be necessary. Preferred embodiments of the present invention include the rectal administration of GDV comprising retroviral vectors that contain genes encoding colon cancer antigens, self and/or foreign MHC, or immune modulators.

Nasal administration avoids first pass metabolism, and gastric acid and enzymatic degradation, and is convenient. In a preferred embodiment, nasal administration is useful for GDV administration wherein the GDV causes the expression of a polypeptide with properties as described above. Conversely, such administration can cause local irritation, and absorption can be dependent upon the state of the nasal mucosa.

Pulmonary administration also avoids first pass metabolism, and gastric acid and enzymatic degradation, and is convenient. Further, pulmonary administration permits localized actions that minimize systemic side effects and the dosage required for effectiveness, and there can be rapid onset of action and self-medication. Conversely, at times only a small portion of the administered composition reaches the brochioli/alveoli, there can be local irritation, and overdosing is possible. Further, patient cooperation and understanding is preferred, and the propellant for dosing may have toxic effects. Preferred embodiments of the present invention include the pulmonary administration of GDV comprising retroviral vectors that contain genes encoding IgA or IgE for the treatment of

conditions such as asthma, hay fever, allergic alveolitis or fibrosing alveolitis, the CFTR gene for the treatment of cystic fibrosis, and protease and collagenous inhibitors such as α-1-antitrypsin for the treatment of emphysema. Alternatively, many of the same types of polypeptides listed above for oral administration may be used.

Ophthalmic administration provides local action, and permit prolonged action where the administration is via inserts. Further, avoids first pass metabolism, and gastric acid and enzymatic degradation, and permits self-administration via the use of eye-drops or contact lens-like inserts. Conversely, the administration is not always efficient, because the administration induces tearing. Preferred embodiments of the present invention include the ophthalmic administration of GDV comprising retroviral vectors that contain genes encoding IgA or IgE for the treatment of hay fever conjunctivitis or vernal and atomic conjunctivitis; and ophthalmic administration of retroviral vectors encoding genes encoding melanoma specific antigens (such as high molecular weight-melanoma associated antigen), self and/or foreign MHC, or immune modulators. For example, GDV expressing inhibitors of angiogenesis can be used for treatment of diabetic lesions of the eye.

Transdermal administration permits rapid cessation of treatment and prolonged action leading to good compliance. Further, local treatment is possible, and avoids first pass metabolism, and gastric acid and enzymatic degradation. Conversely, such administration may cause local irritation, is particularly susceptible to tolerance development, and is typically not preferred for highly potent compositions. Preferred embodiments of the present invention include the transdermal administration of GDV comprising retroviral vectors that contain genes encoding IgA or IgE for the treatment of conditions such as atopic dermatitis and other skin allergies; and transdermal administration of retroviral vectors encoding genes encoding melanoma specific antigens (such as high molecular weight-melanoma associated antigen), self and/or foreign MHC, immune modulators, or the types of polypeptides discussed above.

Vaginal administration provides local treatment and one preferred route for hormonal administration. Further, such administration avoids first pass metabolism, and gastric acid and enzymatic degradation, and is preferred for administration of compositions wherein the GDV exhibit peptides. Preferred embodiments of the present invention include the vaginal administration of GDV comprising retroviral vectors that contain genes encoding self and/or foreign MHC, or immune modulators. other preferred embodiments include the vaginal administration of genes encoding the components of sperm such as histone, flagellin, etc., to promote the production of sperm-specific antibodies and thereby prevent pregnancy. This effect may be reversed, and/or pregnancy in some women may be enhanced, by delivering retroviral vectors encoding immunoglobulin antisense genes, which genes interfere with the production of sperm-specific antibodies.

Intravesical administration permits local treatment for urogenital problems, avoiding systemic side effects and avoiding first pass metabolism, and gastric acid and enzymatic degradation. Conversely, the method requires urethral catheterization and requires a highly skilled staff. Preferred embodiments of intravesical administration include the administration of GDV encoding antitumor genes such as a prodrug activation gene such as kinase (*see* co-pending or various immunomodulatory molecules such as cytokines.

Endoscopic retrograde cystopancreatography (ERCP) (goes through the mouth; does not require piercing of the skin) takes advantage of extended gastroscopy, and permits selective access to the biliary tract and the pancreatic duct. Conversely, the method requires a highly skilled staff, and is unpleasant for the patient.

Preferably, the method of administration is oral administration, which is an aseptic but nonspecific method of administration that passes through the stomach and liver and does not require the presence of any medical personnel for administration. Alternatively, the administration is effected through other non-traumatic routes such as buccal/sublingual, nasal, transdermal, vaginal, rectal, ophthalmic, pulmonary and interotic (preferably buccal/sublingual, nasal, vaginal or rectal) that are also aseptic routes that do not require the presence of any medical personnel for administration. Other suitable non-traumatic routes of administration include gastroscopy, ECRP and colonoscopy, which do not require full operating procedures and hospitalization, but may also require the presence of medical personnel.

In accordance with the non-traumatic administration the present invention, the GDVs may be complexed with a polycationic molecule to provide polycation-assisted non-traumatic administration. Such a method of gene delivery facilitates delivery of a gene via mediation by a physical particle comprised of multiple components that augment the efficiency and specificity of the gene transfer. In particular, polycationic molecules, such as polylysine and histone, have been shown to neutralize the negative charges on a nucleic acid molecule and to condense the molecule into a compact form. This form of molecule is transferred with high efficiency in cells, apparently through the endocytic pathway. The uptake in expression of the nucleic acid molecule in the host cell results after a series of steps, as follows: (1) attachment to cell surface; (2) cell entry via endocytosis or other mechanisms; (3) cytoplasmic compartment entry following endosome release; (4) nuclear transport; and (5) expression of the nucleic acid molecule carried by the GDVs. In a further preferred embodiment, multi-layer technologies are applied to the polycation-nucleic acid molecule complex to facilitate completion of one or more of these steps. For example, a ligand such as asialoglycoprotein, transferrin, and immunoglobulin may be added to the complex to facilitate binding of the cell complex to the cell surface, an endosomal disruption component (*e.g.*, a viral protein, a fusogenic peptide such as the n-terminus of the influenza virus hemaglutinin or an inactivated virus) is added to facilitate the release of DNA from the

endosome, or a nuclear protein (or a peptide containing a nuclear localization signal) is added to facilitate the transport of the DNA into the nucleus. In a further preferred embodiment, the composition comprising the complex includes inactivated adenovirus particles (Curiel, D.T., et al., *PNAS 88*: 8850-8854, 1991; Cristiano, R.J., *PNAS 90*: 2122-2126 1993; Cotten, M., et al., *PNAS 89*: 6094-6098 1992; Lozier, J.N., et al., *Human Gene Therapy 5*: 313-322, 1994; Curiel, D.T., et al., *Human Gene Therapy 3*: 147-154, 1992; Plank, C. et al., *Bioconjugate Chem. 3*: 533-539, 1992; Wagner, E. et al., *PNAS 88*: 4255-4259, 1991). The assorted components comprising the multi-layer complex may be varied as desired, so that the specificity of the complex for a given tissue, or the gene expressed from the GDV, may be varied to better suit a particular disease or condition.

A feature of this embodiment of the invention is that the complex tends to range in size from 80-100 nm (Wagner, E., *supra*), which permits easy access into the endosome vesicle, whose average size is 100-200 nm, and the condensed particle size is not dependent on the molecular weight of the nucleic acid molecule, with a 48 kb DNA molecule transferred into target cells with the same efficiency as a smaller size DNA construct (Cotten, M., 1992).

The GDV may also be administered in combination with the use of *ex vivo* procedures, provided the *ex vivo* procedure results in non-traumatic administration. Such *ex vivo* procedures are particularly advantageous for use with vaginal administration, for example to induce immune responses. Such *ex vivo* procedures include physical and chemical methods of uptake of GDVs into host cells via methods such as calcium phosphate precipitation (Dubensky et al., *PNAS 81*:7529-7533, 1984), direct microinjection of DNA into intact target cells (Acsadi et al., *Nature 352*:815-818, 1991), and electroporation whereby cells suspended in a conducting solution are subjected to an intense electric field in order to transiently polarize the membrane, allowing entry of macromolecules. Other procedures suitable for use with both *in vivo* and *ex vivo* include the use of DNA bound to ligand, DNA linked to an inactive adenovirus (Cotton et al., *PNAS 89*: 6094, 1990), lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991), polycation compounds such as polylysine, receptor specific ligands, liposomes entrapping recombinant GDV, spheroplast fusion whereby *E. coli* containing GDV constructs are stripped of their outer cell walls and fused to animal cells using polyethylene glycol and viral transduction, (Cline et al., *Pharmac. Ther. 29*:69, 1985; and Friedmann et al., *Science 244*:1275, 1989), and DNA ligand (Wu et al, *J. of Biol. Chem. 264*:16985-16987, 1989), as well as psoralen inactivated viruses such as Sendai or Adenovirus. These cells then become the GDVs of the invention.

Alternatively, as noted above, the construct may be carried by a virus such as vaccinia, sindbis or corona virus. Further, methods for introducing a GDV comprising a

retroviral vector into a host cell are described in more detail in an application entitled "Recombinant Retroviruses".

In an *ex vivo* context, the transduced cells are administered into the animal, and monitored for gene expression, or otherwise screened for desired activity. Protocols vary depending on the tissue cells and activity chosen. Briefly, in one embodiment, a recombinant GDV carrying a sequence, the expression of which inhibits MHC class I presentation, is transformed into tissue cells. Preferably, 10^5, 10^6, 10^7, 10^8, to 10^9 tissue cells are transformed. The cells are cultured, and transformed cells may be selected by antibiotic resistance. Cells are assayed for gene expression by Western blot and FACS analysis, or other means. Cells that can be transformed include, but are not limited to, fibroblast cells, bone marrow cells, endothelial cells, keratinocytes, hepatocytes, and thyroid follicular cells.

Within another embodiment of the present invention, wherein the GDV comprise Vector Producing Cells (also termed "VCLs" or "producer cells"), methods are provided for destroying pathogenic agents in an animal, comprising administering the VCLs to the animal in order to destroy the pathogenic agent. One difficulty with the administration of VCLs however, is that in certain instances a very potent immune response may result, thus making such therapy feasible for only a very short term (<2 weeks). Therefore, within preferred embodiments of the invention the immune response against VCLs may be minimized by selecting packaging cell lines made from autologous or HLA-matched human cells. In addition, in order to further limit the immune response against viral structural proteins expressed by the VCLs, the cells may be enclosed in a structure, such as a bead or a bag, which has a semi-permeable membrane, allowing vector particles to diffuse into the animal, but preventing host immune cells from passing through the membrane and thereby generating an immune response. Methods which decrease the immune response allow additional time for *in vivo* transduction to occur, and thus improves the therapy. In each case, the VCL is preferably destroyed by treatment with acyclovir or ganciclovir after it has accomplished its role in the *in vivo* transduction of cells.

Within another embodiment of the invention, patients with metastatic, disseminated cancer may also be treated according to the methods of the present invention. For instance, primary pancreatic carcinomas or colorectal carcinomas that have metastasized to, for example, the liver, may be treated with a viral vector or VCL of the present invention by oral administration. Tumors in the lung or colon may similarly be accessed by pulmonary or rectal administration. Bladder cancer tumors can be treated by introducing GDV into the bladder through the urethra. Tumor cells which have been transduced *in vivo* by, for example, a vector which expresses HSVTK, may then be destroyed by administration of acyclovir or ganciclovir to the patient, giving rise to an augmented anti-tumor response in the presence of cytokines which may be present due to the second GDV in the combination..

Within preferred embodiments of the invention, in addition to administration of a cytotoxic gene or gene products (*e.g.*, HSVTK) as described above, a variety of additional therapeutic compositions may be co-administered or sequentially administered to a warm-blooded animal, in order to inhibit or destroy a pathogenic agent. Such therapeutic compositions may be administered directly, or, within other embodiments, expressed from independent GDVs. Alternatively, a single vector which directs the expression of both a cytotoxic gene or gene product, and a gene which encodes the therapeutic composition (*e.g.*, a non-vector derived gene as discussed above) may be administered to the warm-blooded animal, in order to inhibit or destroy a pathogenic agent. Within a particularly preferred embodiment, vectors or VCLs which deliver and express both the HSVTK gene and a gene coding for an immune accessory molecule, such as human γ-IFN, may be administered to the patient, preferably followed or with another therapeutic vector (*e.g.*, encoding a second cytokine, such as IL-2). In such a construct, one gene may be expressed from the vector LTR and the other may utilize an additional transcriptional promoter found between the LTRs, or may be expressed as a polycistronic mRNA, possibly utilizing an internal ribosome binding site. After *in vivo* gene transfer, the patient's immune system is activated due to the expression of γ-IFN and/or IL-2. After this has occurred, the overall tumor burden itself may be reduced by treating the patient with acyclovir or ganciclovir, allowing more effective immune attack of the tumor. Infiltration of the dying tumor with inflammatory cells, in turn, increases immune presentation and further improves the patient's immune response against the tumor.

Thus, in some embodiments, the GDVs can be administered in such a fashion such that the GDV can either (a) transduce a normal, healthy cell and transform the cell into a producer of a therapeutic protein or other substance which is secreted systemically or (b) transduce an abnormal or defective cell, transforming the cell into a normal functioning phenotype. A composition containing a GDV may also be administered at different sites, the composition may also include multiple GDVs , and the composition also may contain a high titer of virus, where the GDV is a virus.

III. Nucleic Acid Molecules

A nucleic acid molecule non-traumaticly administered to an animal in accordance with the present invention does not naturally occur in the GDV that carries it, and is neither inert nor generally harmful to the animal, but rather provides some desirable benefit, typically an ability to fight a disease or other pathogenic agent. As used herein, "pathogenic agent" refers to a cell that is responsible for a disease state. Representative examples of pathogenic agents include tumor cells, autoreactive immune cells, hormone secreting cells, cells which lack a function that they would normally have, cells that have an

additional inappropriate gene expression which does not normally occur in that cell type, and cells infected with bacteria, viruses, or other intracellular parasites. In addition, as used herein "pathogenic agent" may also refer to a cell that over-expresses or inappropriately expresses a retroviral vector (*e.g.*, in the wrong cell type), or that has become tumorigenic due to inappropriate insertion into a host cell's genome.

A wide variety of nucleic acid molecules may be carried by the GDV of the present invention. Examples of such nucleic acid molecules include genes and other nucleic acid molecules that encode a substance, as well as biologically active nucleic acid molecules such as inactivating sequences that incorporate into a specified intracellular nucleic acid molecule and inactivate that molecule. A nucleic acid molecule is biologically active when the molecule itself provides the desired benefit without requiring the expression of a substance. For example, the biologically active nucleic acid molecule may be an inactivating sequence that incorporates into a specified intracellular nucleic acid molecule and inactivates that molecule, or the molecule may be a tRNA, rRNA or mRNA that has a configuration that provides a binding capability.

Substances include proteins (*e.g.*, antibodies including single chain molecules), immunostimulatory molecules (such as antigens) immunosuppressive molecules, blocking agents, and palliatives (such as toxins, antisense ribonucleic acids, ribozymes, enzymes, anti-angiogenesis compounds and other material capable of inhibiting a function of a pathogenic agent). Anti-angiogenesis compounds can be used in the treatment of a variety of disorders including cancer and diabetic lesions, such as diabetic eye lesions. Substances also include cytokines, and various polypeptides or peptide hormones, and their agonists or antagonists, where these hormones can be derived from tissues such as the pituitary, hypothalamus, kidney, endothelial cells, liver, pancreas, bone, hemopoetic marrow, and adrenal. Such substances can be used for induction of growth, regression of tissue, suppression of immune responses, apoptosis, gene expression, blocking receptor-ligand interaction, immune responses and can be used as treatment for certain anemias, diabetes, infections, high blood pressure, abnormal blood chemistry or chemistries (*e.g.*, elevated blood cholesterol, deficiency of blood clotting factors, elevated LDL with lowered HDL). Such substances can also be used to control levels of Alzheimer associated amyloid protein, bone erosion/calcium deposition, and levels of various metabolites such as steroid hormones, purines, and pyrimidines.

Within the present invention, "capable of inhibiting a function" means that the palliative either directly inhibits the function or indirectly does so, for example, by converting an agent present in the cells from one which would not normally inhibit a function of the pathogenic agent to one which does. Examples of such functions for viral diseases include adsorption, replication, gene expression, assembly, and exit of the virus from infected cells.

Examples of such functions for cancerous diseases include cell replication, susceptibility to external signals (*e.g.*, contact inhibition), and lack of production of anti-oncogene proteins.

Within one embodiment of the present invention, a method is provided for administration of various GDVs, such as eukaryotic viral cDNA expression vectors, which direct the expression of a palliative as a DNA molecule. Within another embodiment of the present invention, a method is provided for administration of various GDVs which direct the expression of a palliative as an RNA molecule.

Representative examples of palliatives that act directly to inhibit the growth of cells include toxins such as ricin (Lamb et al., *Eur. J. Biochem. 148*:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem. 198*:723-732, 1991; Evensen et al., *J. of Biol. Chem. 266*:6848-6852, 1991; Collins et al., *J. of Biol. Chem. 265*:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc. 309*:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem. 260*:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature 306*:551-557, 1983; Sanchez & Holmgren, *PNAS 86*:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem. 255*:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther. 21*:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J. 203*:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys. 200*:418-425, 1980, Irvin, *Arch. Biochem. & Biophys. 169*:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS 84*:4364-4368, 1987; Jackson et al., *Microb. Path. 2*:147-153, 1987), and Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem. 262*:8707-8711, 1987).

Within other aspects of the invention, the GDV carries a gene specifying a product which is not in itself toxic, but when processed or modified by a protein, such as a protease specific to a viral or other pathogen, is converted into a toxic form. For example, the recombinant retrovirus GDV could carry a gene encoding a proprotein chain, which becomes toxic upon processing by the HIV protease. More specifically, a synthetic inactive proprotein form of the toxic ricin or diphtheria A chains could be cleaved to the active form by arranging for the HIV virally encoded protease to recognize and cleave off an appropriate "pro" element.

Within yet another aspect of the invention, the GDV directs the expression of a substance capable of activating an otherwise inactive precursor into an active inhibitor of a pathogenic agent, or a conditional toxic palliative, which are palliatives that are toxic for the cell expressing the pathogenic condition. As will be evident to one of skill in the art given the disclosure provided herein, a wide variety of inactive precursors may be converted into active inhibitors of a pathogenic agent. For example, antiviral nucleoside analogues such as AZT or ddI are metabolized by cellular mechanisms to the nucleotide triphosphate form in order to specifically inhibit retroviral reverse transcriptase, and thus viral replication (Furmam et al., *Proc. Natl. Acad. Sci. USA 83*:8333-8337, 1986). Recombinant viral vectors which direct the expression of a gene product (*e.g.*, a protein) such as Herpes

Simplex Virus Thymidine Kinase (HSVTK) or Varicella Zoster Virus Thymidine Kinase (VZVTK) which assists in metabolizing antiviral nucleoside analogues to their active form are therefore useful in activating nucleoside analogue precursors (*e.g.*, AZT or ddC) into their active form. AZT or ddI therapy will thereby be more effective, allowing lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, will be made more efficacious.

Within one embodiment of the invention, the HSVTK gene may be expressed under the control of a constitutive macrophage or T-cell-specific promoter, and introduced into macrophage or T-cells. Constitutive expression of HSVTK results in more effective metabolism of nucleotide analogues such as AZT or ddI to their biologically active nucleotide triphosphate form, and thereby provides greater efficacy, delivery of lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, may also be utilized within the context of the present invention.

Within a related aspect of the present invention, a GDV directs the expression of a substance that activates another compound with little or no cytotoxicity into a toxic product in the presence of a pathogenic agent, thereby effecting localized therapy to the pathogenic agent. In this case, expression of the gene product from the GDV is limited to situations wherein an entity associated with the pathogenic agent, such as an intracellular signal identifying the pathogenic state, is present, thereby avoiding destruction of nonpathogenic cells. This cell-type specificity may also be conferred at the level of infection, by targeting GDV carrying the vector to cells having or being susceptible to the pathogenic condition.

Within a related aspect of the present invention, a GDV directs the expression of a gene product(s) that activates a compound with little or no cytotoxicity into a toxic product. Briefly, a wide variety of gene products which either directly or indirectly activate a compound with little or no cytotoxicity into a toxic product may be utilized within the context of the present invention. Representative examples of such gene products include HSVTK and VZVTK which selectively monophosphorylate certain purine arabinosides and substituted pyrimidine compounds, converting them to cytotoxic or cytostatic metabolites. More specifically, exposure of the drugs ganciclovir, acyclovir, or any of their analogues (*e.g.*, FIAC, DHPG) to HSVTK, phosphorylates the drug into its corresponding active nucleotide triphosphate form.

For example, within one embodiment of the invention, the GDV directs the expression of the herpes simplex virus thymidine kinase ("HSVTK") gene downstream, and under the transcriptional control of an HIV promoter (which is known to be transcriptionally silent except when activated by HIV tat protein). Briefly, expression of the tat gene product in human cells infected with HIV and carrying the GDV causes increased production of HSVTK. The cells (either *in vitro* or *in vivo*) are then exposed to a drug such as ganciclovir, acyclovir or its analogues (FIAC, DHPG). As noted above, these drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms. Acyclovir triphosphates inhibit cellular polymerases in general, leading to the specific destruction of cells expressing HSVTK in transgenic mice (see Borrelli et al., *Proc. Natl. Acad. Sci. USA 85*:7572, 1988). Those cells containing the recombinant vector and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs.

Within one embodiment of the invention, expression of a conditionally lethal HSVTK gene may be made even more HIV-specific by including cis-acting elements in the transcript ("CRS/CAR"), which require an additional HIV gene product, rev, for optimal activity (Rosen et al., *Proc. Natl. Acad. Sci. USA 85*:2071, 1988). More generally, cis elements present in mRNAs have been shown in some cases to regulate mRNA stability or translatability. Sequences of this type (*i.e.*, post-transcriptional regulation of gene expression) may be used for event- or tissue-specific regulation of vector gene expression. In addition, multimerization of these sequences (*i.e.*, rev-responsive "CRS/CAR" or tat-responsive "TAR" elements for HIV) may be utilized in order to generate even greater specificity.

In a manner similar to the preceding embodiment, GDVs may be generated which carry a gene for phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. Such genes may have no equivalent in mammalian cells, and might come from organisms such as a virus, bacterium, fungus, or protozoan. Representative examples include: *E. coli* guanine phosphoribosyl transferase ("gpt") gene product, which converts thioxanthine into thioxanthine monophosphate (see Besnard et al., *Mol. Cell. Biol. 7*:4139-4141, 1987); alkaline phosphatase, which will convert inactive phosphorylated compounds such as mitomycin phosphate and doxorubicin-phosphate to toxic dephosphorylated compounds; fungal (*e.g.*, *Fusarium oxysporum*) or bacterial cytosine deaminase which will convert 5-fluorocytosine to the toxic compound 5-fluorouracil (Mullen, *PNAS 89*:33, 1992); carboxypeptidase G2 which will cleave the glutamic acid from para-N-bis (2-chloroethyl) aminobenzoyl glutamic acid, thereby creating a toxic benzoic acid mustard; and Penicillin-V amidase, which will convert phenoxyacetamide derivatives of doxorubicin and melphalan to toxic compounds. Conditionally lethal gene products of this type have application to many presently known

purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation in order to become effective cytotoxic agents. The conditionally lethal gene product could also metabolize a nontoxic drug, which is not a purine or pyrimidine analogue, to a cytotoxic form (see Searle et al., *Brit. J. Cancer 53*:377-384, 1986).

These kinds of conditional activation of an inactive precursor into an active product in cells may be achieved using a GDV such as a viral vector including an adeno-associated viral vector, and those with a shorter term effect, *e.g.*, an adenovirus vector and others mentioned below. Such a vector is capable of efficiently entering cells and expressing proteins encoded by the vector over a period of time from a couple of days to a month or so. This period of time should be sufficient to allow killing of pathogenic cells. In addition, physical methods of gene transfer may be utilized in a similar manner.

Additionally, in the instance where the target pathogen is a mammalian virus, the GDV may be constructed to take advantage of the fact that mammalian viruses in general tend to have "immediate early" genes, which are necessary for subsequent transcriptional activation of other viral promoter elements. Gene products of this nature are excellent candidates for intracellular signals (or "identifying agents") of viral infection. Thus, conditionally lethal genes transcribed from transcriptional promoter elements that are responsive to such viral "immediate early" gene products could specifically kill cells infected with any particular virus. Additionally, since the human α and β interferon promoter elements are transcriptionally activated in response to infection by a wide variety of nonrelated viruses, the introduction of vectors expressing a conditionally lethal gene product like HSVTK, for example, from these viral-responsive elements (VREs) could result in the destruction of cells infected with a variety of different viruses.

In another embodiment of the invention, methods are provided for producing substances such as inhibitor palliatives involving the delivery and expression of defective interfering viral structural proteins, which inhibit viral assembly. In this context, GDV codes for defective gag, pol, env or other viral particle proteins or peptides which inhibit in a dominant fashion the assembly of viral particles. Such inhibition occurs because the interaction of normal subunits of the viral particle is disturbed by interaction with the defective subunits.

One way of increasing the effectiveness of inhibitory palliatives is to express inhibitory genes, such as viral inhibitory genes, in conjunction with the expression of genes which increase the probability of infection of the resistant cell by the virus in question. The result is a nonproductive "dead-end" event which would compete for productive infection events. In the specific case of HIV, a GDV may be non-traumaticly administered that inhibits HIV replication (by expressing anti-sense tat, etc., as described above) and also overexpress proteins required for infection, such as CD4. In this way, a relatively small

number of vector-infected HIV-resistant cells act as a "sink" or "magnet" for multiple nonproductive fusion events with free virus or virally infected cells.

In another embodiment of the invention, methods are provided for the expression substances such as inhibiting peptides or proteins specific for viral protease. Viral protease cleaves the viral gag and gag/pol proteins into a number of smaller peptides. Failure of this cleavage in all cases leads to complete inhibition of production of infectious retroviral particles. The HIV protease is known to be an aspartyl protease, and these are known to be inhibited by peptides made from amino acids from protein or analogues. GDV that inhibit HIV will express one or multiple fused copies of such peptide inhibitors.

The approaches discussed above should be effective against many virally linked diseases, cancers, or other pathogenic agents.

Within still other embodiments of the invention, a GDV is provided that expresses a palliative, wherein the palliative has a membrane anchor and acts as an anti-tumor agent(s). Such a palliative may be constructed, for example, as an anti-tumor agent - membrane anchor fusion protein. Briefly, the membrane anchor aspect of the fusion protein may be selected from a variety of sequences, including, for example, the transmembrane domain of well known molecules. Generally, membrane anchor sequences are regions of a protein that bind the protein to a membrane. Customarily, there are two types of anchor sequences that attach a protein to the outer surface of a cell membrane: (1) transmembrane regions that span the lipid bilayer of the cell membrane, and interact with the hydrophobic center region (proteins containing such regions are referred to integral membrane proteins), and (2) domains which interact with an integral membrane protein or with the polar surface of the membrane (such proteins are referred to as peripheral, or extrinsic, proteins).

Membrane anchors for use within the present invention may contain transmembrane domains which span the membrane one or more times. For example, in glycophorin and guanylyl cyclase, the membrane binding region spans the membrane once, whereas the transmembrane domain of rhodopsin spans the membrane seven times, and that of the photosynthetic reaction center of Rhodopseudomonas viridis spans the membrane eleven times (*see* Ross et al., *J. Biol. Chem. 257*:4152, 1982; Garbers, *Pharmac. Ther. 50*:337-345, 1991; Engelman et al., *Proc. Natl. Acad. Sci. USA 77*:2023, 1980; Heijne and Manoil, *Prot. Eng. 4*:109-112, 1990). Regardless of the number of times the protein crosses the membrane, the membrane spanning regions typically have a similar structure. More specifically, the 20 to 25 amino-acid residue portion of the domain that is located inside the membrane generally consists almost entirely of hydrophobic residues (*see* Eisenberg et al., *Ann. Rev. Biochem. 53*:595-623, 1984). For example, 28 of the 34 residues in the membrane spanning region of glycophorin are hydrophobic (*see* Ross et al., *supra*; Tomita et al., *Biochemistry 17*:4756-4770, 1978). In addition, although structures such as beta sheets and barrels do occur, the membrane spanning regions typically have an alpha helical structure, as

determined by X-ray diffraction, crystallography and cross-linking studies (*see* Eisenberg et al., *supra*; Heijne and Manoil, *supra*). The location of these transmembrane helices within a given sequence can often be predicted based on hydrophobicity plots. Stryer et al., *Biochemistry*, 3rd. ed. 304, 1988. Particularly preferred membrane anchors for use within the present invention include naturally occurring cellular proteins (that are non-immunogenic) which have been demonstrated to function as membrane signal anchors (such as glycophorin).

Within a preferred embodiment of the present invention, a DNA sequence is provided which encodes a membrane anchor - gamma interferon fusion protein. Within one embodiment, this fusion protein may be constructed by genetically fusing the sequence which encodes the membrane anchor of the gamma-chain of the Fc receptor, to a sequence which encodes gamma-interferon.

In yet another aspect, the GDV provides a therapeutic effect by encoding one or more ribozymes (RNA enzymes) (Haseloff and Gerlach, *Nature 334*:585, 1989) which will cleave, and hence inactivate, RNA molecules corresponding to a pathogenic function. Since ribozymes function by recognizing a specific sequence in the target RNA and this sequence is normally 12 to 17 bp, this allows specific recognition of a particular RNA sequence corresponding to a pathogenic state, such as HIV tat, and toxicity is specific to such pathogenic state. Additional specificity may be achieved in some cases by making this a conditional toxic palliative, as discussed above.

In still another aspect, the GDV comprises a biologically active nucleic acid molecule that is an antisense sequence (an antisense sequence may also be encoded by a nucleic acid sequence and then produced within a host cell via transcription). In preferred embodiments, the antisense sequence is selected from the group consisting of sequences which encode influenza virus, HIV, HSV, HPV, CMV, and HBV. The antisense sequence may also be an antisense RNA complementary to RNA sequences necessary for pathogenicity. Alternatively, the biologically active nucleic acid molecule may be a sense RNA (or DNA) complementary to RNA sequences necessary for pathogenicity.

More particularly, the biologically active nucleic acid molecule may be an antisense sequence. Briefly, antisense sequences are designed to bind to RNA transcripts, and thereby prevent cellular synthesis of a particular protein, or prevent use of that RNA sequence by the cell. Representative examples of such sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick, *Arch. Biochem. & Biophys. 253*:214-220, 1987; Bzik et al., *PNAS 84*:8360-8364, 1987), antisense HER2 (Coussens et al., *Science 230*:1132-1139, 1985), antisense ABL (Fainstein et al., *Oncogene 4*:1477-1481, 1989), antisense Myc (Stanton et al., *Nature 310*:423-425, 1984) and antisense *ras*, as well as antisense sequences which block any of the enzymes in the nucleotide biosynthetic pathway.

In addition, within a further embodiment of the invention antisense RNA may be utilized as an anti-tumor agent in order to induce a potent Class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences are believed to induce the increased expression of interferons (including gamma-interferon), due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

In another embodiment, the substances of the invention include a surface protein that is itself therapeutically beneficial. For example, in the particular case of HIV, expression of the human CD4 protein specifically in HIV-infected cells may be beneficial in two ways:

1. Binding of CD4 to HIV env intracellularly could inhibit the formation of viable viral particles much as soluble CD4 has been shown to do for free virus, but without the problem of systematic clearance and possible immunogenicity, since the protein will remain membrane bound and is structurally identical to endogenous CD4 (to which the patient should be immunologically tolerant).

2. Since the CD4/HIV env complex has been implicated as a cause of cell death, additional expression of CD4 (in the presence of excess HIV-env present in HIV-infected cells) leads to more rapid cell death and thus inhibits viral dissemination. This may be particularly applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractility to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4 on their cell surfaces).

Still further aspects of the present invention relate to the non-traumatic administration of a GDV capable of immunostimulation. The ability to recognize and defend against foreign pathogens is essential to the function of the immune system. In particular, the immune system must be capable of distinguishing "self" from "nonself" (*i.e.*, foreign), so that the defensive mechanisms of the host are directed toward invading entities instead of against host tissues. Cytolytic T lymphocytes (CTLs) are typically induced, or stimulated, by the display of a cell surface recognition structure, such as a processed, pathogen-specific peptide, in conjunction with a MHC class I or class II cell surface protein.

Diseases suitable to treatment include viral infections such as HIV, HBV and HPV, cancers such as melanomas, renal carcinoma, breast cancer, ovarian cancer and other cancers, and heart disease.

In one embodiment, the invention provides methods for stimulating a specific immune response and inhibiting viral spread by using GDVs that direct the expression of an antigen or modified form thereof in susceptible target cells, wherein the antigen is capable of either (1) initiating an immune response to the viral antigen or (2) preventing the viral spread by occupying cellular receptors required for viral interactions. Expression of the protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the recombinant viral vector is preferably designed to express a modified form of the antigen which will stimulate an immune response and which has reduced pathogenicity relative to the native antigen. This immune response is achieved when cells present antigens in the correct manner, *i.e.*, in the context of the MHC class I and/or II molecules along with accessory molecules such as CD3, ICAM-1, ICAM-2, LFA-1, or analogs thereof (*e.g.*, Altmann et al., *Nature 338*:512, 1989). In accordance with a preferred embodiment, cells infected with Sindbis viral vectors are expected to do this efficiently because they closely mimic genuine viral infection and (a) are able to infect non-replicating cells; (b) do not integrate into the host cell genome; and (c) are not associated with any life threatening diseases.

This embodiment of the invention has a further advantage over other systems that might be expected to function in a similar manner, in that the presenter cells are fully viable and healthy, and no other viral antigens (which may well be immunodominant) are expressed. This presents a distinct advantage since the antigenic epitopes expressed can be altered by selective cloning of sub-fragments of the gene for the antigen into the recombinant Sindbis virus, leading to responses against immunogenic epitopes which may otherwise be overshadowed by immunodominant epitopes. Such an approach may be extended to the expression of a peptide having multiple epitopes, one or more of the epitopes being derived from different proteins. Further, this aspect of the invention allows efficient stimulation of cytotoxic T lymphocytes (CTL) directed against antigenic epitopes, and peptide fragments of antigens encoded by sub-fragments of genes, through intracellular synthesis and association of these peptide fragments with MHC Class I molecules. This approach may be utilized to map major immunodominant epitopes for CTL induction.

An immune response can also be achieved by transferring to an appropriate immune cell (such as a T lymphocyte) (a) the gene for the specific T-cell receptor that recognizes the antigen of interest (in the context of an appropriate MHC molecule if necessary), (b) the gene for an immunoglobulin which recognizes the antigen of interest, or (c) the gene for a hybrid of the two which provides a CTL response in the absence of the MHC context. Thus the GDV may be used as an immunostimulant, immunomodulator, or vaccine, etc.

In the particular case of disease caused by HIV infection, where immunostimulation is desired, the antigen generated from the recombinant retroviral genome

is of a form which will elicit either or both an HLA class I- or class II-restricted immune response. In the case of HIV envelope antigen, for example, the antigen is preferably selected from gp 160, gp 120, and gp 41, which have been modified to reduce their pathogenicity. In particular, the selected antigen is modified to reduce the possibility of syncytia, to avoid expression of epitopes leading to a disease enhancing immune response, to remove immunodominant, but haplotype-specific epitopes or to present several haplotype-specific epitopes, and allow a response capable of eliminating cells infected with most or all strains of HIV. The haplotype-specific epitopes can be further selected to promote the stimulation of an immune response within an animal which is cross-reactive against other strains of HIV. Antigens from other HIV genes or combinations of genes, such as gag, pol, rev, vif, nef, prot, gag/pol, gag prot, etc., may also provide protection in particular cases.

HIV is only one example. This approach should be effective against many virally linked diseases or cancers where a characteristic antigen (which does not need to be a membrane protein) is expressed, such as in HPV and cervical carcinoma, HTLV-I-induced leukemias, prostate-specific antigen (PSA) and prostate cancer, mutated p53 and colon carcinoma and melanoma, melanoma specific antigens (MAGEs), and melanoma, mucin and breast cancer.

In accordance with the immunostimulation aspects of the invention, the substances of the present invention may also include "immunomodulatory factors," many of which are set forth above. Immunomodulatory factors refer to factors that, when manufactured by one or more of the cells involved in an immune response, or, which when added exogenously to the cells, causes the immune response to be different in quality or potency from that which would have occurred in the absence of the factor. The factor may also be expressed from a non-GDV derived gene, but the expression is driven or controlled by the GDV. The quality or potency of a response may be measured by a variety of assays known to one of skill in the art including, for example, *in vitro* assays which measure cellular proliferation (*e.g.*, ^{3}H thymidine uptake), and *in vitro* cytotoxic assays (*e.g.*, which measure ^{51}Cr release) (*see*, Warner et al., *AIDS Res. and Human Retroviruses* 7:645-655, 1991). Immunomodulatory factors may be active both *in vivo* and *ex vivo*.

Representative examples of such factors include cytokines, such as IL-1, IL-2 (Karupiah et al., *J. Immunology 144*:290-298, 1990; Weber et al., *J. Exp. Med. 166*:1716-1733, 1987; Gansbacher et al., *J. Exp. Med. 172*:1217-1224, 1990; U.S. Patent No. 4,738,927), IL-3, IL-4 (Tepper et al., *Cell 57*:503-512, 1989; Golumbek et al., *Science 254*:713-716, 1991; U.S. Patent No. 5,017,691), IL-5, IL-6 (Brakenhof et al., *J. Immunol. 139*:4116-4121, 1987; WO 90/06370), IL-7 (U.S. Patent No. 4,965,195) , IL-8, IL-9, IL-10, IL-11, IL-12, IL-13 (*Cytokine Bulliten*, Summer 1994), particularly IL-2, IL-4, IL-6, IL-12, and IL-13, alpha interferon (Finter et al., *Drugs 42*(5):749-765, 1991; U.S. Patent No. 4,892,743; U.S. Patent No. 4,966,843; WO 85/02862; Nagata et al., *Nature* 284:316-320,

1980; Familletti et al., *Methods in Enz. 78*:387-394, 1981; Twu et al., *Proc. Natl. Acad. Sci. USA 86*:2046-2050, 1989; Faktor et al., *Oncogene 5*:867-872, 1990), beta interferon (Seif et al., *J. Virol. 65*:664-671, 1991), gamma interferons (Radford et al., *The American Society of Hepatology* 20082015, 1991; Watanabe et al., *PNAS 86*:9456-9460, 1989; Gansbacher et al., *Cancer Research 50*:7820-7825, 1990; Maio et al., *Can. Immunol. Immunother. 30*:34-42, 1989; U.S. Patent No. 4,762,791; U.S. Patent No. 4,727,138), G-CSF (U.S. Patent Nos. 4,999,291 and 4,810,643), GM-CSF (WO 85/04188), tumor necrosis factors (TNFs) (Jayaraman et al., *J. Immunology 144*:942-951, 1990), CD3 (Krissanen et al., *Immunogenetics 26*:258-266, 1987), ICAM-1 (Altman et al., *Nature 338*:512-514, 1989; Simmons et al., *Nature 331*:624-627, 1988), ICAM-2, LFA-1, LFA-3 (Wallner et al., *J. Exp. Med. 166*(4):923-932, 1987), MHC class I molecules, MHC class II molecules, B7.1-.3, β 2-microglobulin (Parnes et al., *PNAS 78*:2253-2257, 1981), chaperones such as calnexin, MHC linked transporter proteins or analogs thereof (Powis et al., *Nature 354*:528-531, 1991). Within one preferred embodiment, the gene encodes gamma-interferon.

An example of an immunomodulatory factor cited above is a member of the B7 family of molecules (*e.g.*, B7.1-.3 costimulatory factor). Briefly, activation of the full functional activity of T cells requires two signals. One signal is provided by interaction of the antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to as costimulation, is delivered to the T cell by antigen presenting cells. The second signal is required for interleukin-2 (IL-2) production by T cells, and appears to involve interaction of the B7.1-.3 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., *J. Exp. Med., 173*:721-730, 1991a and *J. Exp. Med., 174*:561-570, 1991). Within one embodiment of the invention, B7.1-.3 may be introduced into tumor cells in order to cause costimulation of $CD8^+$ T cells, such that the $CD8^+$T cells produce enough IL-2 to expand and become fully activated. These $CD8^+$ T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7.1-.3 factor, and, for example, an immunogenic HBV core protein, may be made utilizing methods which are described herein. Cells transduced with these vectors will become more effective antigen presenting cells. The HBV core-specific CTL response will be augmented from the fully activated $CD8^+$ T cell via the costimulatory ligand B7.1-.3.

The choice of which immunomodulatory factor to include within a GDV may be based upon known therapeutic effects of the factor, or, experimentally determined. For example, a known therapeutic effector in chronic hepatitis B infections is alpha interferon. This has been found to be efficacious in compensating a patient's immunological deficit, and thereby assisting recovery from the disease. Alternatively, a suitable immunomodulatory factor may be experimentally determined. Briefly, blood samples are first taken from patients

with a hepatic disease. Peripheral blood lymphocytes (PBLs) are restimulated *in vitro* with autologous or HLA matched cells (*e.g.*, EBV transformed cells) that have been transduced with a GDV which directs the expression of an immunogenic portion of a hepatitis antigen and the immunomodulatory factor. These stimulated PBLs are then used as effectors in a CTL assay with the HLA matched transduced cells as targets. An increase in CTL response over that seen in the same assay performed using HLA matched stimulator and target cells transduced with a vector encoding the antigen alone, indicates a useful immunomodulatory factor. Within one embodiment of the invention, the immunomodulatory factor gamma interferon is particularly preferred.

The present invention also include immunogenic portions of desired antigens. For example, various immunogenic portions of the HBV S antigens may be combined in order to present an immune response when administered by one of the GDVs described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S antigen open reading frame of HBV, particular combinations of antigens may be preferred for non-traumatic administration in particular geographic regions. Briefly, epitopes that are found in all human hepatitis B virus S samples are defined as determinant "*a*". Mutually exclusive subtype determinants however have also been identified by two-dimensional double immunodiffusion (Ouchterlony, *Progr. Allergy* 5:1, 1958). These determinants have been designated "*d*" or "*y*" and "*w*" or "*r*" (LeBouvier, *J. Infect.* *123*:671, 1971; Bancroft et al., *J. Immunol. 109*:842, 1972; Courouce et al., *Bibl. Haematol.* *42*:1-158, 1976). The immunological variability is due to single nucleotide substitutions in two areas of the hepatitis B virus S antigen open reading frame resulting in the following amino acid changes: (1) exchange of lysine-122 to arginine in the hepatitis B virus S antigen open reading frame causes a subtype shift from *d* to *y*, and (2) exchange of arginine-160 to lysine causes the shift from subtype *r* to *w*. In black Africa, subtype *ayw* is predominant, whereas in the U.S. and northern Europe the subtype adw_2 is more abundant (*Molecular Biology of the Hepatitis B Virus*, McLachlan (ed.), CRC Press, 1991). As will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for non-traumatic administration which is appropriate to the particular hepatitis B virus subtype which is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, preferably, by sequencing the S antigen open reading frame of HBV virus isolated from individuals within that region.

Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected liver tissue. The polymerase protein consists of at least two domains: the amino terminal domain encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase

and RNase H activity. Immunogenic portions of HBV pol may be determined utilizing methods utilizing GDVs administered in order to generate an immune response within an animal, preferably a warm-blooded animal. Similarly, other HBV antigens such as ORF 5 and ORF 6, (Miller et al., *Hepatology 9*:322-327, 1989), may be expressed utilizing GDVs as described herein.

As noted above, at least one immunogenic portion of a hepatitis B antigen can be incorporated into a GDV. The immunogenic portion(s) which are incorporated into the GDV may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long, and may include the entire antigen. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature 351*:290, 1991). From this analysis, peptides may be synthesized and used as targets in an *in vitro* cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays, and proliferation assays.

Within one embodiment of the present invention, at least one immunogenic portion of a hepatitis C antigen can be incorporated into a GDV. Preferred immunogenic portion(s) of hepatitis C may be found in the C and NS3-NS4 regions since these regions are the most conserved among various types of hepatitis C virus (Houghton et al., *Hepatology 14*:381-388, 1991). Particularly preferred immunogenic portions may be determined by a variety of methods. For example, as noted above for the hepatitis B virus, identification of immunogenic portions of the polypeptide may be predicted based upon amino acid sequence. Briefly, various computer programs which are known to those of ordinary skill in the art may be utilized to predict CTL epitopes. For example, CTL epitopes for the HLA A2.1 haplotype may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature 351:290, 1991)*. From this analysis, peptides are synthesized and used as targets in an *in vitro* cytotoxic assay.

Within another aspect of the present invention, methods are provided for destroying hepatitis B carcinoma cells comprising the step of non-traumaticly administering to a warm-blooded animal a GDV which directs the expression of an immunogenic portion of antigen X, such that an immune response is generated. Sequences which encode the HBxAg may readily be obtained by one of skill in the art given the disclosure provided herein. Briefly, within one embodiment of the present invention, a 642 bp Nco I-Taq I is recovered from ATCC 45020, and inserted into GDVs as described above for other hepatitis B antigens.

The X antigen, however, is a known transactivator which may function in a manner similar to other potential oncogenes (*e.g.*, E1A). Thus, it is generally preferable to first alter the X antigen such that the gene product is non-tumorigenic before inserting it into

a GDV. Various methods may be utilized to render the X antigen non-tumorigenic including, for example, by truncation, point mutation, addition of premature stop codons, or phosphorylation site alteration. Within one embodiment, the sequence or gene of interest which encodes the X antigen is truncated. Truncation may produce a variety of fragments, although it is generally preferable to retain greater than or equal to 50% of the encoding gene sequence. In addition, it is necessary that any truncation leave intact some of the immunogenic sequences of the gene product. Alternatively, within another embodiment of the invention, multiple translational termination codons may be introduced into the gene. Insertion of termination codons prematurely terminates protein expression, thus preventing expression of the transforming portion of the protein.

The X gene or modified versions thereof may be tested for tumorigenicity in a variety of ways. Representative assays include tumor formation in nude mice, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice.

Within another aspect of the present invention, methods are provided for destroying hepatitis C carcinoma cells comprising the step of non-traumaticly administering to a warm-blooded animal a GDV which directs the expression of an immunogenic portion of a hepatitis C antigen. Preferred immunogenic portion(s) of a hepatitis C antigen may be found in the polypeptide which contains the Core antigen and the NS1-NS5 regions (Choo et al., *Proc. Natl. Acad. Sci. USA 88*:2451-2455, 1991). Particularly preferred immunogenic portions may be determined by a variety of methods. For example, as noted above preferred immunogenic portions may be predicted based upon amino acid sequence. Briefly, various computer programs which are known to those of ordinary skill in the art may be utilized to predict CTL epitopes. For example, CTL epitopes for the HLA A2.1 haplotype may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature 351*:290, 1991). Another method that may also be utilized to predict immunogenic portions is to determine which portion has the property of CTL induction in mice utilizing retroviral vectors (*see*, Warner et al., *AIDS Res. and Human Retroviruses* 7:645-655, 1991). As noted within Warner et al., CTL induction in mice may be utilized to predict cellular immunogenicity in humans. Preferred immunogenic portions may also be deduced by determining which fragments of the polypeptide antigen or peptides are capable of inducing lysis by autologous patient lymphocytes of target cells (*e.g.*, autologous EBV-transformed lymphocytes) expressing the fragments after vector transduction of the corresponding genes.

Preferred immunogenic portions may also be selected in the following manner. Briefly, blood samples from a patient with a target disease, such as HCV, are analyzed with antibodies to individual HCV polypeptide regions (*e.g.*, HCV core, E1, E2/SNI and NS2-NS5 regions), in order to determine which antigenic fragments are present in the patient's serum. In patients treated with alpha interferon to give temporary remission, some antigenic determinants will disappear and be supplanted by endogenous antibodies to

the antigen. Such antigens are useful as immunogenic portions within the context of the present invention (Hayata et al., *Hepatology 13*:1022-1028, 1991; Davis et al., *N. Eng. J. Med. 321*:1501-1506, 1989).

Additional immunogenic portions of a chosen antigen, such as those from the hepatitis B or C virus, may be obtained by truncating the coding sequence. For example, with HBV the following sites may be truncated: Bst UI, Ssp I, Ppu MI, and Msp I (Valenzuela et al., *Nature 280*:815-19, 1979; Valenzuela et al., *Animal Virus Genetics: ICN/UCLA Symp. Mol. Cell Biol.*, 1980, B. N. Fields and R. Jaenisch (eds.), pp. 57-70, New York: Academic). Further methods for determining suitable immunogenic portions as well as methods are also described below in the context of hepatitis C.

With respect to the treatment of HBV, particularly preferred immunogenic portions for incorporation into GDVs include HBeAg, HBcAg, and HBsAgs. Further, more than one immunogenic portion (as well as immunomodulatory factors, if desired) may be incorporated into the GDV. For example, within one embodiment a GDV may be prepared which directs the co-expression of both an immunogenic portion of the hepatitis B antigen, as well as an immunogenic portion of the hepatitis C polypeptide. Such constructs may be non-traumaticly administered in order to prevent or treat acute and chronic hepatitis infections of either type B or C. Similarly, within other embodiments, a GDV may be prepared which directs the co-expression of both an immunogenic portion of the hepatitis B X antigen, as well as an immunogenic portion of the hepatitis C polypeptide. Such a construct may similarly be non-traumaticly administered in order to treat hepatocellular carcinoma that is associated with either hepatitis B or C. In addition, because those individuals chronically infected with hepatitis B and C are at higher risk for developing hepatocellular carcinoma, such a vector may also be utilized as a prophylactic treatment for the disease.

Immunogenic portions may also be selected by other methods. For example, the HLA A2.1/K^b transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T-cell receptor repertoire recognizes the same antigenic determinants recognized by human T-cells. In both systems, the CTL response generated in the HLA A2.1/K^b transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al., *J. Exp. Med. 173*:1007-1015, 1991; Vitiello et al., *Abstract of Molecular Biology of Hepatitis B Virus Symposia*, 1992).

Immunogenic proteins of the present invention may also be manipulated by a variety of methods known in the art, in order to render them more immunogenic. Representative examples of such methods include: adding amino acid sequences that correspond to T helper epitopes; promoting cellular uptake by adding hydrophobic residues; by forming particulate structures; or any combination of these (*see generally*, Hart, op. cit.,

Milich et al., *Proc. Natl. Acad. Sci. USA 85*:1610-1614, 1988; Willis, *Nature 340*:323-324, 1989; Griffiths et al., *J. Virol. 65*:450-456, 1991).

The present invention also includes compositions and methods for treating, as well as vaccines for preventing, various feline diseases, including for example feline leukemia virus ("FeLV") and feline immunodeficiency virus ("FIV") infections. This viruses are discussed more fully in co-pending .

Feline leukemia virus (FeLV) is a retrovirus of the oncornavirus subfamily. FeLV is presently believed to exist in three subgroups - A, B or C - which are differentiated by their envelope antigens gp70 and p15E. FeLV is also comprised of a number of core antigens, including p15, p12, p27, and p10, which are highly conserved for all subgroups of FeLV (*see* Geering et al., *Vir. 36*:678-680, 1968; Hardy et al., *JAVMA 158*:1060-1069, 1971; Hardy et al., *Science 166*:1019-1021, 1969). Within one embodiment of the invention, the GDV directs the expression of at least one portion of a feline leukemia virus antigen selected from the group consisting of p15*gag*, p12*gag*, p27*gag*, p10*gag*, p14*pol*, p80*pol*, p46*pol*, gp70*env*, and p15*env*. Within a particularly preferred embodiment, the GDV directs the expression of gp85*env*. Sequences which encode these antigens may be readily obtained given the disclosure provided herein (*see* Donahue et al., *J. Vir. 62*(3):722-731, 1988; Stewart et al., *J. Vir. 58*(3):825-834, 1986; Kumar et al., *J. Vir. 63*(5):2379-2384, 1989; Elder et al., *J. Vir. 46*(3):871-880, 1983; Berry et al., *J. Vir. 62*(10):3631-3641, 1988; Laprevotte et al., *J. Vir. 50*(3):884-894, 1984).

Feline immunodeficiency virus (FIV) has been classified as a retrovirus of the lentivirus subfamily, based upon the magnesium requirement for reverse transcriptase (RT) and the morphology of viral particles (*see* Pederesen et al., *Science 235*:790-793, 1987). The feline immunodeficiency virus is morphologically and antigenically distinct from other feline retroviruses, including feline leukemia virus, type C oncorna virus (RD-114), and feline syncytium-forming virus (FeSFV) (*see* Yamamoto et al., "Efficacy of experimental FIV vaccines, (Abstract), First International Conference of Feline Immunodeficiency Virus Researchers, University of California, Davis, CA, Sep. 4-7, 1991). Within one embodiment of the invention, the GDV directs the expression of at least one immunogenic portion of an feline immunodeficiency virus antigen selected from the group consisting of p15*gag*, p24*gag*, p10*gag*, p13*pol*, p62*pol*, p15*pol* and p36*pol*. Within a particularly preferred embodiment, the GDV directs the expression of gp68*env*, gp27*env* and *rev*. Within the context of the present invention, "*rev*" is understood to refer to the antigen corresponding to the *rev* open reading frame (*see*, Phillips et al., First International Conference, *supra*). Sequences which encode these antigens may be readily obtained by one of skill in the art given the disclosure provided herein (*see* Phillips et al., *J. Vir. 64*(10):4605-4613, 1990; Olmsted et al., *PNAS 86*:2448-2452, 1989; Talbott et al., *PNAS 86*:5743-5747, 1989).

Still other examples include a GDV which directs the expression of a non-tumorigenic, altered ras (ras*) gene. Briefly, the ras* gene is an attractive target because it is causally linked to the neoplastic phenotype, and indeed may be necessary for the induction and maintenance of tumorigenesis in a wide variety of distinct cancers, such as pancreatic carcinoma, colon carcinoma and lung adenocarcinoma. In addition, ras* genes are found in pre-neoplastic tumors, and therefore immune intervention therapy may be applied prior to detection of a malignant tumor.

Normal ras genes are non-tumorigenic and ubiquitous in all mammals. They are highly conserved in evolution and appear to play an important role in maintenance of the cell cycle and normal growth properties. The normal ras protein is a G-protein which binds GTP and has GTPase activity, and is involved in transmitting signals from the external milieu to the inside of the cell, thereby allowing a cell to respond to its environment. Ras* genes on the other hand alter the normal growth regulation of neoplastic cells by uncoupling cellular behavior from the environment, thus leading to the uncontrolled proliferation of neoplastic cells. Mutation of the ras gene is believed to be an early event in carcinogenesis (Kumar et al., "Activation of ras Oncogenes Preceding the Onset of Neoplasia," *Science 248*:1101-1104, 1990), which, if treated early, may prevent tumorigenesis.

Ras* genes occur in a wide variety of cancers, including for example, pancreatic, colon, and lung adenocarcinomas. However, the spectrum of mutations occurring in the ras* genes found in a variety of cancers is quite limited. These mutations alter the GTPase activity of the ras protein by converting the normal on/off switch to a constitutive ON position. Tumorigenic mutations in ras* occur primarily (*in vivo*) in only 3 codons: 12, 13 and 61. Codon 12 mutations are the most prevalent in both human and animal tumors.

Within another embodiment of the present invention, a GDV is provided which directs the expression of an altered p53 (p53*) gene. Briefly, p53 is a nuclear phosphoprotein which was originally discovered in extracts of transformed cells, and thus was initially classified as an oncogene (Linzer and Levine, *Cell 17*:43-52, 1979; Lane and Crawford, *Nature 278*:261-263, 1979). It was later discovered that the original p53 cDNA clones were mutant forms of p53 (Hinds et al., *J. Virol. 63*:739-746, 1989). It now appears that p53 is a tumor suppressor gene, which negatively regulates the cell cycle, and that mutation of this gene may lead to tumor formation. Of colon carcinomas that have been studied, 75%-80% show a loss of both p53 alleles, one through deletion, and the other through point mutation. Similar mutations are found in lung cancer, and in brain and breast tumors.

The majority of p53 mutations (*e.g.*, $p53^{*1}$, $p53^{*2}$, etc.) are clustered between amino-acid residues 130 to 290 (*see* Levine et al., *Nature 351*:453-456, 1991; *see also* the following references which describe specific mutations in more detail: Baker et al.,

Science 244:217-221, 1989; Nigro et al., *Nature 342*:705-708, 1989 (p53 mutations cluster at four "hot spots" which coincide with the four highly conserved regions of the genes and these mutations are observed in human brain, breast, lung and colon tumors); Vogelstein, *Nature 348*:681-682, 1990; Takahashi et al., *Science 246*:491-494, 1989; Iggo et al., *Lancet 335*:675-679, 1990; James et al., *Proc. Natl. Acad. Sci. USA 86*:2858-2862, 1989; Mackay et al., *Lancet 11*:1384-1385,1988; Kelman et al., *Blood 74*:2318-2324, 1989; Malkin et al., *Science 250*:1233-1238, 1990; Baker et al., *Cancer Res. 50*:7717-7722, 1991; Chiba et al., *Oncogene 5*:1603-1610, 1990 (pathogenesis of early stage non-small cell lung cancer is associated with somatic mutations in the p53 gene between codons 132 to 283); Prosser et al., *Oncogene 5*:1573-1579, 1990 (mutations in the p53 gene coding for amino acids 126 through 224 were identified in primary breast cancer); Cheng and Hass, *Mol. Cell. Biol. 10*:5502-5509, 1990; Bartek et al., *Oncogene 5*:893-899, 1990; Rodrigues et al., *Proc. Natl. Acad. Sci. USA 87*:7555-7559, 1990; Menon et al., *Proc. Natl. Acad. Sci. USA 87*:5435-5439, 1990; Mulligan et al., *Proc. Natl. Acad. Sci. USA 87*:5863-5867, 1990; and Romano et al., *Oncogene 4*:1483-1488, 1990 (identification of a p53 mutation at codon 156 in human osteosarcoma derived cell line HOS-SL)).

Certain alterations of the p53 gene may be due to certain specific toxins. For example, Bressac et al. (*Nature 350*:429-431, 1991) describes specific G to T mutations in codon 249, in patients affected with hepatocellular carcinoma. One suggested causative agent of this mutation is aflatoxin B_1, a liver carcinogen which is known to be a food contaminant in Africa.

Four regions of the gene that are particularly affected occur at residues 132-145, 171-179, 239-248, and 272-286. Three "hot spots" of particular interest occur at residues 175, 248 and 273 (Levine et al., *Nature 351*:453-456, 1991). These alterations as well as others which are described above result in the production of protein(s) which contain novel coding sequence(s). The novel proteins encoded by these sequences may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding regions may be utilized to destroy tumorigenic cells containing the altered sequence ($p53^*$).

Within another embodiment of the present invention, a GDV is provided which directs the expression of an altered Rb (Rb^*) gene. Briefly, retinoblastoma is a childhood eye cancer associated with the loss of a gene locus designated Rb, which is located in chromosome band 13q14. A gene from this region has been cloned which produces a nuclear phosphoprotein of about 110 kd (Friend et al., *Nature 323*:643, 1986; Lee et al., *Science 235*:1394, 1987; and Fung et al., *Science 236*:1657, 1987).

Rb is believed to be a negative regulator of cellular proliferation, and has a role in transcriptional control and cell-cycle regulation. Rb binds to at least seven proteins found in the nucleus, and in particular, appears to be involved with a cellular transcription factor which has been designated both E2F (Bagchi et al., *Cell 62*:659-669, 1990) and DRTF

(Shivji and La Thangue, *Mol. Cell. Biol. 11*:1686-1695, 1991). Rb is believed to restrict cellular growth by sequestering a variety of nuclear proteins involved in cellular proliferation.

Deletions within the Rb gene have been detected which evidence that the Rb gene may be responsible for tumorigenicity. These deletions include, for example, a deletion in exon 21 in a prostate cancer and bladder cancer cell line (Bookstein et al., *Science 247*:712-715, 1990; Horowitz et al., *Science 243*:937, 1989), a deletion of exon 16 in a small-cell carcinoma of the lung (Shew et al., *Cell Growth and Diff. 1*:17, 1990), and a deletion between exons 21 and 27 (Shew et al., *Proc. Natl. Acad. Sci. USA 87*:6, 1990). Deletion of these exons results in the production of a protein containing a novel coding sequence at the junction of the deleted exons. This novel protein coding sequence may be used as a marker of tumorigenic cells, and an immune response directed against this novel coding region may eliminate tumorigenic cells containing the Rb exon deletion.

Within another embodiment of the present invention, a GDV is provided which directs the expression of an altered gene which causes Wilms' tumor. Briefly, Wilms' tumor is typically found in children younger than 16 years of age. One child in 10,000 will develop this tumor, which comprises approximately 5% of childhood cancers. The tumor usually presents itself as a large abdominal mass which is surrounded by a fibrous pseudocapsule. Approximately 7% of the tumors are multifocal in one kidney, and 5.4% are involved with both kidneys. The Wilms' tumor gene has been localized to chromosome 11p13, and a cDNA clone (wt1) has been isolated that is characteristic of a tumor suppressor gene (Call et al., *Cell 60*:509, 1990; Gessler et al., *Nature 343*:744, 1990; Rose et al., *Cell 60*:495, 1990; and Haber et al., *Cell 61*:1257, 1990). The wt1 gene encodes a protein which contains four zinc fingers and a glutamine and proline rich amino terminus. Such structures are believed to be associated with transcriptional and regulatory functions.

Mutations of the Wilms' tumor gene include the insertion of lysine, threonine, and serine between the third and forth zinc fingers. A wt1 protein which contains such insertions does not bind to the EGR-1 site. A second alternative mutation results in the insertion of about 17 amino acids in the region immediately NH_2-terminal to the zinc finger domain (Madden et al., *Science 253*:1550-1553, 1991; Call et al., *Cell 60*:509, 1990; Gessler et al., *Nature 343*:744, 1990; Rose et al., *Cell 60*:495, 1990; Haber et al., *Cell 61*:1257, 1990; and Buckler et al., *Mol. Cell. Biol. 11*:1707, 1991).

Within another embodiment of the present invention, a GDV is provided which directs the expression of an altered mucin. Mucins are large molecular weight glycoproteins which contain approximately 50% carbohydrate. Polymorphic epithelial mucin (PEM) is a tumor-associated mucin (Girling et al., *Int. J. Cancer 43*:1072-1076, 1989) which is found in the serum of cancer patients. The full-length cDNA sequence has been identified (Gendler et al., *J. Biol. Chem. 265*(25):15286-15293, 1990; Lan et al., *J. Biol. Chem. 265*(25):15294-15299, 1990; and Ligtenberg et al., *J. Biol. Chem. 265*:5573-5578, 1990).

Breast tumors and pancreatic tumors both express a mucin with an identical core sequence, containing a 20 amino-acid tandem repeat (Jerome et al., *Cancer Res. 51*:2908-2916, 1991). CTL lines which have been developed to breast tumors which cross-react with pancreatic tumor targets, and further appear to specifically recognize the specific 20 amino-acid tandem repeat (Jerome et al., *supra*). A sequence encoding one or more of the 20 amino-acid tandem repeats may be expressed by a GDV of the present invention, in order to develop an immune response against tumor cells which contain this sequence.

Within another embodiment of the present invention, a GDV is provided which directs the expression of an altered DCC (deleted in colorectal carcinomas) gene. Briefly, a very common region of allelic loss in colorectal tumors is chromosome 18q, which is lost in more than 70% of carcinomas, and in almost 50% of late adenomas. A presumptive tumor suppressor gene (DCC) from this region has been identified (Fearon et al., 1990), which encodes a protein with significant homology to cell-surface adhesion molecules, such as neural cell-adhesion molecule (NCAM) and contactin (reviewed by Edelman in *Biochem 27*:3533-3543, 1988). This protein is believed to play a role in the development of colorectal tumors, perhaps through alterations in normal cell-cell and/or cell-extracellular matrix interactions.

The DCC gene is expressed in normal colonic mucosa, but its expression is reduced or absent in the majority of colorectal carcinomas (Solomon, *Nature 343*:412-414, 1990). This loss of expression has been associated in some cases with somatic mutations of the DCC gene. A contiguous stretch of DNA comprising 370 kb has been cloned which encodes an approximately 750 amino acid protein (Fearon et al., "Identification of a Chromosome 18q Gene That Is Altered in Colorectal Cancers," *Science 247*:49-56, 1990).

Within another embodiment of the present invention, a GDV is provided which directs the expression of MCC (mutated in colorectal cancer) or APC. Both MCC and APC have been identified as tumor suppressor genes (Kinzler et al., *Science 251*:1366-1370, 1991) which undergo mutation in familial adenomatous polyposis (FAP). FAP is believed to be the most common autosomal dominant disease which leads to cancer, and it affects at least 1 in 5,000 individuals in the United States (Nishiho et al., *Science 253*:665-669, 1991). Affected individuals usually develop hundreds to thousands of adenomatous polyps of the colon and rectum, which may progress to carcinoma. Gardner's syndrome ("GS," a variant of FAP) presents desmoid tumors, osteomas, and other neoplasms together with multiple adenomas of the colon and rectum. This proliferation is believed to be induced by loss or inactivation of the familial adenomatous polyposis gene (and in particular, MCC and APC) which is found on chromosome 5q.

For example, in Nishiho et al. (*supra*), the following germ line mutations of the APC gene were found in FAP and GS patients: (1) Codon 280, a serine to stop mutation (in a patient with mandibular osteoma), (2) codon 302, an arginine to stop mutation in two

separate patients, one with a desmoid tumor, (3) codon 414, an arginine to cysteine mutation in a patient with mandibular osteoma, and (5) codon 713, a serine to stop mutation in another patient with mandibular osteoma (Nishiho et al., *Science 253*:665-669, 1991). In addition, six point mutations were identified in MCC codon numbers 12, 145, 267, 490, 506, and 698, as well as an additional 4 somatic mutations in APC (codons number 289, 332, 438, and 1338).

Within other embodiments of the invention, a GDV is provided which directs the expression of an altered receptor which is functionally locked or stuck in an "ON" or "OFF" mode. Briefly, many cellular receptors are involved in cell growth by monitoring the external environment and signaling the cell to respond appropriately. If either the monitoring or signaling mechanisms fail, the cell will no longer respond to the external environment and may exhibit uncontrolled growth. Many different receptors or receptor-like structures may function as altered cellular components, including, for example, neu and mutated or altered forms of the thyroid hormone receptor, the PDGF receptor, the insulin receptor, the Interleukin receptors (*e.g.*, IL-1, -2, -3, etc. receptors), or the CSF receptors, such as the G-CSF, GM-CSF, or M-CSF receptors.

For example, neu (also referred to as the Human Epidermal Growth Factor Receptor "HER" or the Epidermal Growth Factor "EGF" receptor) is an altered receptor which is found in at least 28% of women with breast cancer. A cDNA clone which encodes this protein has been isolated (Slamon et al., *Science 244*:707-712, 1989; Slamon et al., *Cancer Cells 7*:371-380, 1989; Shih et al., *Nature 290*:261, 1981). This clone encodes a protein that has extracellular, transmembrane, and intracellular domains (Schechter, *Nature 312*:513, 1984; Coussens et al., *Science 230*:1132, 1985) and thus is believed to encode the neu receptor.

Studies of the rat neu gene isolated from chemically induced neuroglioblastoma cells indicate that it contains a single mutation at position 664 from valine to glutamic acid (Bargmann et al., *EMBO J. 7*:2043, 1988). In other studies, baby rats which were treated with N-ethyl-N-nitrosourea developed malignant tumors of the nervous system. All 47 trigeminal schwannomas and 12 neurinomas which developed carried a T to A transversion at position 664 of the neu gene (Nikitin et al., *Proc. Natl. Acad. Sci USA 88*:9939-9943, 1991)..

Other altered receptors may also be expressed by GDVs in order to destroy selected tumor cells. For example, a deletion in chromosome 3p21-p25 has been associated with small-cell lung carcinomas (Leduc et al., *Am. J. Hum. Genet. 44*:282-287, 1989). A deletion is believed to occur in the ERBAb gene which otherwise codes for a DNA-binding thyroid hormone receptor (THR).

Alterations in receptors as described above result in the production of protein(s) (or receptors) containing novel coding sequence(s). The novel protein(s) encoded

by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding region(s) may be utilized to destroy tumorigenic cells containing the altered sequence(s) or gene(s).

If the altered cellular component is associated with making the cell tumorigenic, then, it is necessary to make the altered cellular component non-tumorigenic. For example, within one embodiment, the sequence or gene of interest which encodes the altered cellular component is truncated in order to render the gene product non-tumorigenic. The gene encoding the altered cellular component may be truncated to a variety of sizes, although it is preferable to retain as much as possible of the altered cellular component. In addition, it is necessary that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational termination codons may be introduced into the gene which encodes the altered cellular component, downstream of the immunogenic region. Insertion of termination codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

Within one embodiment, the ras* gene is truncated in order to render the ras* protein non-tumorigenic. Briefly, the carboxy-terminal amino acids of ras* functionally allow the protein to attach to the cell membrane. Truncation of these sequences renders the altered cellular component non-tumorigenic. Preferably, the ras* gene is truncated in the purine ring formation, for example around the sequence which encodes amino acid number 110. The ras* gene sequence may be truncated such that as little as about 20 amino acids (including the altered amino acid(s) are encoded by the GDV, although preferably, as many amino acids as possible should be expressed (while maintaining non-tumorigenicity).

Within another embodiment, the p53* protein is modified by truncation in order to render the cellular component non-tumorigenic. As noted above, not all mutations of the p53 protein are tumorigenic, and therefore, not all mutations would have to be truncated. Nevertheless, within a preferred embodiment, p53* is truncated to a sequence which encodes amino acids 100 to 300, thereby including all four major "hot spots."

Other altered cellular components which are oncogenic may also be truncated in order to render them non-tumorigenic. For example, both neu and bcr/abl may be truncated in order to render them non-tumorigenic. Non-tumorigenicity may be confirmed by assaying the truncated altered cellular component as described above.

It should be noted, however, that if the altered cellular component is only associated with non-tumorigenic cells in general, and is not required or essential for making the cell tumorigenic, then it is not necessary to render the cellular component non-tumorigenic. Representative examples of such altered cellular components which are not tumorigenic include Rb*, ubiquitin*, and mucin*.

As noted above, in order to generate an appropriate immune response, the altered cellular component must also be immunogenic. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes often possess an immunogenic amphipathic alpha-helix component. In general, however, it is preferable to determine immunogenicity in an assay. Representative assays include an ELISA which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells such as gamma-interferon assays, IL-2 production assays, and proliferation assays. A particularly preferred method for determining immunogenicity is the CTL assay.

Once a sequence encoding at least one anti-tumor agent has been obtained, it is preferable to ensure that the sequence encodes a non-tumorigenic protein. Various assays are known and may easily be accomplished which assess the tumorigenicity of a particular cellular component. Representative assays include tumor formation in nude mice or rats, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice.

For this and many other aspects of the invention, tumor formation in nude mice or rats is a particularly important and sensitive method for determining the tumorigenicity of an anti-tumor agent. Nude mice lack a functional cellular immune system (*i.e.*, do not possess CTLs), and therefore provide a useful *in vivo* model in which to test the tumorigenic potential of cells. Normal non-tumorigenic cells do not display uncontrolled growth properties if injected into nude mice. However, transformed cells will rapidly proliferate and generate tumors in nude mice. Briefly, in one embodiment the GDV is delivered to syngeneic murine cells, followed by administration into nude mice. The mice are visually examined for a period of 2 to 8 weeks after administration in order to determine tumor growth. The mice may also be sacrificed and autopsied in order to determine whether tumors are present. (Giovanella et al., *J. Natl. Cancer Inst. 48*:1531-1533, 1972; Furesz et al., "Tumorigenicity testing of cell lines considered for production of biological drugs," *Abnormal Cells*, New Products and Risk, Hopps and Petricciani (eds.), Tissue Culture Association, 1985; and Levenbook et al., *J. Biol. Std. 13*:135-141, 1985). Tumorigenicity may also be assessed by visualizing colony formation in soft agar (MacPherson and Montagnier, *Vir. 23*:291-294, 1964). Briefly, one property of normal non-tumorigenic cells is "contact inhibition" (*i.e.*, cells will stop proliferating when they touch neighboring cells). If cells are plated in a semi-solid agar support medium, normal cells rapidly become contact inhibited and stop proliferating, whereas tumorigenic cells will continue to proliferate and form colonies in soft agar.

Transgenic animals, such as transgenic mice, may also be utilized to assess the tumorigenicity of an anti-tumor agent (*e.g.*, Stewart et al., *Cell 38*:627-637, 1984; Quaife et al., *Cell 48*:1023-1034, 1987; and Koike et al., *Proc. Natl. Acad. Sci. USA 86*:5615-5619, 1989). In transgenic animals, the gene of interest may be expressed in all tissues of the

animal. This unregulated expression of the transgene may serve as a model for the tumorigenic potential of the newly introduced gene.

In addition to tumorigenicity studies, it is generally preferable to determine the toxicity of the toxic palliatives, such as anti-tumor agent(s), prior to non-traumatic administration. A variety of methods well known to those of skill in the art may be utilized to measure such toxicity, including for example, clinical chemistry assays which measure the systemic levels of various proteins and enzymes, as well as blood cell volume and number.

Cell mediated and humoral responses may also be induced against a pathogenic agent, particularly viral and bacterial diseases, by non-traumatic administration of immunogenic portion(s) as discussed above. Briefly, immunogenic portions carrying relevant epitopes can be produced in a number of known ways (Ellis and Gerety, *J. Med. Virol. 31*:54-58, 1990), including chemical synthesis (Bergot et al., *Applied Biosystems Peptide Synthesizer User Bulletin No. 16, 1986*, Applied Biosystems, Foster City, California) and DNA expression in recombinant systems, such as the insect-derived baculovirus system (Doerfler, *Current Topics in Immunology 131*:51-68, 1986), mammalian-derived systems (such as CHO cells) (Berman et al., *J. Virol. 63*:3489-3498, 1989), yeast-derived systems (McAleer et al., *Nature 307*:178-180), and prokaryotic systems (Burrel et al., *Nature 279*:43-47, 1979).

The present invention also provides a GDV capable of immune down-regulation. Specific down-regulation of inappropriate or unwanted immune responses, such as in autoimmune or pseudo-autoimmune diseases such as chronic hepatitis, diabetes, rheumatoid arthritis, graft vs. host disease and Alzheimer's, or in transplants of heterologous tissue such as bone marrow, can be engineered using immune-suppressive viral gene products, or active portion thereof, which suppress surface expression of transplantation (MHC) antigen. Within the present invention, an "active portion" of a gene product is that fragment of the gene product which must be retained for biological activity. Such fragments or active domains can be readily identified by systematically removing nucleotide sequences from the protein sequence, transforming target cells with the resulting recombinant GDV, and determining MHC class I presentation on the surface of cells using FACS analysis or other immunological assays, such as a CTL assay. These fragments are particularly useful when the size of the sequence encoding the entire protein exceeds the capacity of the viral carrier. Alternatively, the active domain of the MHC antigen presentation inhibitor protein can be enzymatically digested and the active portion purified by biochemical methods. For example, a monoclonal antibody that blocks the active portion of the protein can be used to isolate and purify the active portion of the cleaved protein (Harlow et al., *Antibodies: A Laboratory Manual*, Cold Springs Harbor, 1988).

Within one embodiment, the suppression is effected by specifically inhibiting the activation of display of processed peptides in the context of self MHC molecules along

with accessory molecules such as CD8, intercellular adhesion molecule -1 (ICAM-1), ICAM-2, ICAM-3, leukocyte functional antigen-1 (LFA-1) (Altmann et al., *Nature 338*:521, 1989), the B7.1-.3 molecule (Freeman et al., *J. Immunol. 143*:2714, 1989), LFA-3 (Singer, *Science 255*:1671, 1992; Rao, *Crit. Rev. Immunol. 10*:495, 1991), or other cell adhesion molecules. Antigenic peptide presentation in association with MHC class I molecules leads to CTL activation. Transfer and stable integration of specific sequences capable of expressing products expected to inhibit MHC antigen presentation block activation of T-cells, such as $CD8^+$ CTL, and therefore suppress graft rejection. A standard CTL assay is used to detect this response. Components of the antigen presentation pathway include the 45 Kd MHC class I heavy chain, β_2-microglobulin, processing enzymes such as proteases, accessory molecules, chaperonenes such as calnexin (Gaczynska, *et al.*, *Nature*, *365*: 264-282, 1993), and transporter proteins such as PSF1, TAP1 and TAP 2 (Driscoll, *et al.*, *Nature*, *365*: 262-263, 1993).

In an alternative example, the recombinant GDV directs the expression of a gene product or an active portion of a gene product capable of binding β_2-microglobulin. Transport of MHC class I molecules to the cell surface for antigen presentation requires association with β_2-microglobulin. Thus, proteins that bind β_2-microglobulin and inhibit its association with MHC class I indirectly inhibit MHC class I antigen presentation. Suitable proteins include the H301 gene product. Briefly, the H301 gene, obtained from the human cytomegalovirus (CMV) encodes a glycoprotein with sequence homology to the β_2-microglobulin binding site on the heavy chain of the MHC class I molecule (Browne et al., *Nature 347*:770, 1990). H301 binds β_2-microglobulin, thereby preventing the maturation of MHC class I molecules, and renders transformed cells unrecognizable by cytotoxic T-cells, thus evading MHC class I restricted immune surveillance.

Within another embodiment, the recombinant GDV directs the expression of a protein or active portion of a protein that binds to newly synthesized MHC class I molecules intracellularly. This binding prevents migration of the MHC class I molecule from the endoplasmic reticulum, resulting in the inhibition of terminal glycosylation. This blocks transport of these molecules to the cell surface and prevents cell recognition and lysis by CTL. For instance, one of the products of the E3 gene may be used to inhibit transport of MHC class I molecules to the surface of the transformed cell. More specifically, E3 encodes a 19 kD transmembrane glycoprotein, E3/19K, transcribed from the E3 region of the adenovirus 2 genome. Within the context of the present invention, tissue cells are transformed with a recombinant GDV containing the E3/19K sequence, which upon expression produces the E3/19K protein. The E3/19K protein inhibits the surface expression of MHC class I surface molecules, and cells transformed by the GDV evade an immune response. Consequently, donor cells can be transplanted with reduced risk of graft rejection and may require only a minimal immunosuppressive regimen for the transplant patient. This

allows an acceptable donor-recipient chimeric state to exist with fewer complications. Similar treatments may be used to treat the range of so-called autoimmune diseases, including systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis or chronic hepatitis B infection.

Another alternative method of immunosuppression involves the use of antisense message, ribozyme, or other gene expression inhibitor specific for T-cell clones which are autoreactive in nature. These block the expression of the T-cell receptor of particular unwanted clones responsible for an autoimmune response. The anti-sense, ribozyme, or other gene may be introduced using a viral vector delivery system.

Other proteins, not discussed above, that function to inhibit, suppress or down-regulate MHC class I antigen presentation may also be identified and utilized within the context of the present invention. In order to identify such proteins, in particular those derived from mammalian pathogens (and, in turn, active portions thereof), a recombinant GDV that expresses a protein or an active portion thereof suspected of being capable of inhibiting MHC class I antigen presentation is transformed into a tester cell line, such as BC. The tester cell lines with and without the sequence encoding the candidate protein are compared to stimulators and/or targets in the CTL assay. A decrease in cell lysis corresponding to the transformed tester cell indicates that the candidate protein is capable of inhibiting MHC presentation.

Another alternative method to determine down-regulation of MHC class I surface expression is by FACS analysis. More specifically, cell lines are transformed with a recombinant GDV encoding the candidate protein. After drug selection and expansion, the cells are analyzed by FACS for MHC class I expression and compared to that of non-transformed cells. A decrease in cell surface expression of MHC class I indicates that the candidate protein is capable of inhibiting MHC presentation. This aspect of the present invention is further discussed in a priority application.

Many infectious diseases, cancers, autoimmune diseases, and other diseases involve the interaction of viral particles with cells, cells with cells, or cells with factors. In viral infections, viruses commonly enter cells via receptors on the surface of susceptible cells. In cancers, cells may respond inappropriately or not at all to signals from other cells or factors. In autoimmune disease, there is inappropriate recognition of "self" markers. Within the present invention, such interactions may be blocked by utilizing GDVs that produce, *in vivo*, an analogue to either of the partners in an interaction. Such an analogue is known as a blocking agent.

This blocking action may occur intracellularly, on the cell membrane, or extracellularly. The blocking action of a viral or, in particular, a retroviral GDV carrying a gene for a blocking agent, can be mediated either from inside a susceptible cell or by secreting a version of the blocking protein to locally block the pathogenic interaction.

For example, in the case of HIV, the two agents of interaction are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker would be a GDV expressing either an HIV env analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Delivery of a retroviral vector encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule.

Vector particles leading to expression of HIV env may also be constructed. It will be evident to one skilled in the art which portions are capable of blocking virus adsorption without overt pathogenic side effects (Willey et al., *J. Virol. 62*:139, 1988; Fisher et al., *Science 233*:655, 1986).

Another aspect of the invention involves the delivery of suppressor genes which, when deleted, mutated or not expressed in a cell type, lead to tumorigenesis in that cell type. Reintroduction of the deleted gene by means of a viral vector leads to regression of the tumor phenotype in these cells. Since malignancy can be considered to be an inhibition of cellular terminal differentiation compared with cell growth, the delivery and expression of gene products which lead to differentiation of a tumor should also, in general, lead to regression.

Sequences which encode the above-described altered cellular components may be obtained from a variety of sources. For example, plasmids which contain sequences that encode altered cellular products may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as Advanced Biotechnologies (Columbia, Maryland). Representative examples of plasmids containing some of the above-described sequences include ATCC No. 41000 (containing a G to T mutation in the 12th codon of ras), and ATCC No. 41049 (containing a G to A mutation in the 12th codon).

Alternatively, plasmids which encode normal cellular components may also be obtained from depositories such as the ATCC (*see, for example*, ATCC No. 41001 which contains a sequence which encodes the normal ras protein, ATCC No. 57103 which encodes abl; and ATCC Nos. 59120 or 59121 which encode the bcr locus) and mutated to form the altered cellular component. Methods for mutagenizing particular sites may readily be accomplished using methods known in the art (*see* Sambrook et al., *supra.*, 15.3 *et seq.*). In particular, point mutations of normal cellular components such as ras may readily be accomplished by site-directed mutagenesis of the particular codon, for example, codons 12, 13 or 61.

Other nucleic acid molecules that encode the above-described substances, as well as other nucleic acid molecules that are advantageous for use within the present invention, may be readily obtained from a variety of sources, including for example depositories such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Representative examples include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No 67024 (which contains a sequence which encodes Interleukin-1b), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6).

Molecularly cloned genomes which encode the hepatitis B virus may be obtained from a variety of sources including, for example, the American Type Culture Collection (ATCC, Rockville, Maryland). For example, ATCC No. 45020 contains the total genomic DNA of hepatitis B (extracted from purified Dane particles) (*see* Figure 3 of Blum et al., *TIG* 5(5):154-158, 1989) in the Bam HI site of pBR322 (Moriarty et al., *Proc. Natl. Acad. Sci. USA* 78:2606-2610, 1981). (Note that correctable errors occur in the sequence of ATCC No. 45020.)

Alternatively, cDNA sequences for use with the present invention may be obtained from cells which express or contain the sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligo dT or random primers. The single stranded cDNA may then be amplified by PCR (*see* U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159. See also *PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.), Stockton Press, 1989) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Nucleic acid molecules that are suitable for use with the present invention may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, APB DNA synthesizer model 392 (Foster City, California).

IV. Gene Therapy

One further aspect of the present invention is the non-traumatic administration of vectors and nucleic acid sequences such as those described above to achieve a therapeutic effect or an enhanced therapeutic effect.

As an example of the present invention, a GDV can be used to treat Gaucher disease. Gaucher disease is a genetic disorder that is characterized by the deficiency of the enzyme glucocerebrosidase. This type of therapy is an example of a single gene replacement therapy by providing a deficient cellular enzyme. This enzyme deficiency leads to the accumulation of glucocerebroside in the lysosomes of all cells in the body. However, the disease phenotype is manifested only in the macrophages except in the very rare neuronpathic forms of the disease. The disease usually leads to enlargement of the liver and spleen and lesions in the bones. (For a review see *Science 256*:794, 1992 and *The Metabolic Basis of Inherited Disease*, 6th ed., Scriver, et al., vol. 2, p. 1677). Various approaches for treatment have included supplying exogenous enzyme purified from human placenta using various macrophage targeting techniques including resealed erythrocytes coated with immunoglobulin and liposome technology but the clinical improvements in patients was minimal. Not until the human glucocerebrosidase was purified on an industrial scale and modified for macrophage targeting (commercially known as aglucerase™) has significant clinical improvement been observed. However, this treatment can run an average $765,000 per year per patient (see *Science 256*:794, 1992 for review).

In the context of the present invention, when patients are treated with a gene for a protein that they do not have the capacity to express, there may not only be an antibody response to the protein, but also a cellular immune response to the cells making the protein. This can be obviated or minimized by administering a GDV encoding the glucocerebrosidase in combination with a GDV encoding an immune suppressive gene such as adenoviral E3 protein.

V. Pharmaceutically Acceptable Carriers and Diluents

Pharmaceutically acceptable carriers or diluents are nontoxic to recipients at the dosages and concentrations employed. Preferably, the carrier is a solid carrier suitable for enhancing the administration of an anhydrous GDV in a tablet or capsule.

In an embodiment where one or more, and preferably all, of the GDVs are retroviral vectors, an aqueous suspension containing the GDVs in crude or purified form can be dried by lyophilization or evaporation at ambient temperature. Specifically, lyophilization involves the steps of cooling the aqueous suspension below the glass transition temperature or below the eutectic point temperature of the aqueous suspension, and removing water from the cooled suspension by sublimation to form a lyophilized virus. Briefly, aliquots of the

formulated recombinant virus are placed into an Edwards Refrigerated Chamber (3 shelf RC3S unit) attached to a freeze dryer (Supermodulyo 12K). A multistep freeze drying procedure as described by Phillips et al. (*Cryobiology 18*:414, 1981) is used to lyophilize the formulated recombinant virus, preferably from a temperature of -40°C to -45°C. The resulting composition contains less than 10% water by weight of the lyophilized virus. Once lyophilized, the recombinant virus is stable and may be stored at -20°C to -25°C.

With the evaporative method, water is removed from the aqueous suspension at ambient temperature by evaporation. Within one embodiment, water is removed through spray drying (EP 520,748). Within the spray drying process, the aqueous suspension is delivered into a flow of preheated gas, usually air, whereupon water rapidly evaporates from droplets of the suspension. Spray drying apparatus are available from a number of manufacturers (*e.g.*, Drytec, Ltd., Tonbridge, England; Lab-Plant, Ltd., Huddersield, England). Once dehydrated, the recombinant virus is stable and may be stored at -20°C to -25°C. Within the methods described herein, the resulting moisture content of the dried or lyophilized virus may be determined through use of a Karl-Fischer apparatus (EM Science Aquastar™ V1B volumetric titrator, Cherry Hill, NJ), or through a gravimetric method.

Representative examples of carriers or diluents where the composition is not dry include water, isotonic saline solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin. A preferred composition comprises a vector or recombinant virus in 10 mg/ml mannitol, 1 mg/ml HSA, 20 mM Tris, pH 7.2, and 150 mM NaCl. In this case, since the GDV represents approximately 1 mg of material, it may be less than 1% of high molecular weight material, and less than 1/100,000 of the total material (including water). This composition is stable at -70°C for at least six months. In some cases, it may be necessary to add a weak polycation such as polyprene or DEAE-Dextran at 1-30 μg/ml to improve transduction frequencies.

The pharmaceutically acceptable carrier or diluent may be combined with the GDV to provide a composition either as a liquid solution, or as a solid form (e.g., lyophilized) which may be, but is not preferably, resuspended in a solution prior to administration. In addition the composition may be prepared with suitable carriers or diluents for either oral, nasal or rectal administration or other means appropriate to the composition. The GDV is typically purified to a concentration ranging from 0.25% to 25%, and preferably about 5% to 20% before formulation. Subsequently, after preparation of the composition, where the GDV is a recombinant virus, the recombinant virus will constitute about 10 ng to 1 μg of material per dose, with about 10 times this amount of material present as copurified contaminants. Preferably, the composition is prepared in 0.1-1.0 ml of aqueous solution formulated as described below.

The pharmaceutically acceptable compositions of the present invention may also additionally include factors which stimulate cell division, and hence, uptake and incorporation of a GDV such as a recombinant retroviral vector. Representative examples include Melanocyte Stimulating Hormone (MSH), for melanomas, epidermal growth factor (EGF) for breast or other epithelial carcinomas, and the anesthetic bipuvocaine (or related compounds) for intramuscular injection. Particularly preferred methods and compositions for preserving recombinant viruses are described in U.S. applications entitled "Methods for Preserving Recombinant Viruses".

As noted above, the GDV may direct expression of an immunomodulatory factor in addition to at least one immunogenic portion of a desired antigen. If the GDV, however, does not express an immunomodulatory factor which is a cytokine, this cytokine may be included in the above-described compositions, or may be administered separately (concurrently or subsequently) with the above-described compositions. Briefly, within such an embodiment, the immunomodulatory factor is preferably administered according to standard protocols and dosages as prescribed in *The Physician's Desk Reference*. For example, alpha interferon may be administered at a dosage of 1-5 million units/day for 2-4 months, and IL-2 at a dosage of 10,000-100,000 units/kg of body weight, 1-3 times/day, for 2-12 weeks. Gamma interferon may be administered at dosages of 150,000-1,500,000 units 2-3 times/week for 2-12 weeks.

A preferred composition comprises a vector or recombinant virus in 10 mg/ml mannitol, 1 mg/ml HSA, 20 mM Tris, pH 7.2, and 150 mM NaCl. In this case, since the recombinant vector represents approximately 1 μg of material, it may be less than 1% of high molecular weight material, and less than 1/100,000 of the total material (including water). This composition is stable at -70°C for at least six months.

Preferably, the composition, or a representative sample of the composition, is first non-traumaticly administered to an animal via the desired route, then the animal is tested for biological response. Such testing may include immunological screening assays (*e.g.*, CTL assays, antibody assays) for evidence of immune response to the HIV gene products and the HBV gene product. Based upon such testing, the titers of the GDVs may be adjusted to further enhance the desired effect(s). Next, the composition is non-traumaticly administered to a human being via the appropriate route, followed by screening assays and other testing to determine the effectiveness of the composition.

The pharmaceutically acceptable compositions of the present invention may also additionally include factors which stimulate cell division, and hence, uptake and incorporation of a GDV such as a recombinant retroviral vector. Representative examples include Melanocyte Stimulating Hormone (MSH), for melanomas or epidermal growth factor (EGF) for breast or other epithelial carcinomas.

As noted above, the GDV may direct expression of an immunomodulatory cofactor in addition to the desired substance. If the GDV, however, does not express such an immunomodulatory cofactor, for example a cytokine, the immunomodulatory cofactor may be included in the above-described compositions, or may be administered separately (concurrently or subsequently) with the above-described compositions. Briefly, within such an embodiment, the immunomodulatory cofactor is preferably administered according to standard protocols and dosages as prescribed in *The Physician's Desk Reference*. For example, alpha interferon may be administered at a dosage of 1-5 million units/day for 2-4 months, and IL-2 at a dosage of 10,000-100,000 units/kg of body weight, 1-3 times/day, for 2-12 weeks. Gamma interferon may be administered at dosages of 150,000-1,500,000 units 2-3 times/week for 2-12 weeks.

Within preferred embodiments of the invention, in addition to non-traumatic administration of a cytotoxic gene or gene products (*e.g.*, HSVTK) as described above, a variety of additional therapeutic compositions may be co-non-traumaticly administered or sequentially administered to a warm-blooded animal, in order to inhibit or destroy a pathogenic agent. Such therapeutic compositions may be administered directly, or, within other embodiments, expressed from independent GDVs. Alternatively, a single GDV that directs the expression of both a cytotoxic gene or gene product, and a gene which encodes the therapeutic composition (*e.g.*, a non-vector derived gene as discussed above) may be administered to the warm-blooded animal, in order to inhibit or destroy a pathogenic agent. Within a particularly preferred embodiment, vector or VCLs which deliver and express both the HSVTK gene and a gene coding for an immune accessory molecule, such as human γ-IFN, may be administered to the patient. In such a construct, one gene may be expressed from the vector LTR and the other may utilize an additional transcriptional promoter found between the LTRs, or may be expressed as a polycistronic mRNA, possibly utilizing an internal ribosome binding site. After *in vivo* gene transfer, the patient's immune system is activated due to the expression of c-IFN. After this has occurred, the overall tumor burden itself may be reduced by treating the patient with acyclovir or ganciclovir, allowing more effective immune attack of the tumor. Infiltration of the dying tumor with inflammatory cells, in turn, increases immune presentation and further improves the patient's immune response against the tumor.

Thus, in some embodiments, the GDV can be non-traumaticly administered in such a fashion such that the GDV can either (a) transduce a normal, healthy cell and transform the cell into a producer of a therapeutic protein or other substance which is secreted systemically or (b) transform an abnormal or defective cell, transforming the cell into a normal functioning phenotype.

The GDV may be administered to animals. In preferred embodiments, the animal is a warm-blooded animal, further preferably selected from the group consisting of

mice, chickens, cattle, horses, and humans. Alternatively, the animal may be a cold-blooded animal, preferably selected from the group consisting of fish, aquatic vertebrates, and shellfish.

EXAMPLES

Example 1

Preparation of Retroviral Vector Backbones

Preparation of Retroviral Backbones KT-1 and KT-3B

The Moloney murine leukemia virus (MoMLV) 5' long terminal repeat (LTR) EcoR I-EcoR I fragment, including *gag* sequences, from the N2 vector (Armentano *et al.*, *J. Vir. 61*:1647-1650, 1987; Eglitas *et al.*, *Science 230*:1395-1398, 1985) is ligated into the plasmid SK^+ (Stratagene, La Jolla, CA). The resulting construct is designated N2R5. The N2R5 construct is mutated by site-directed *in vitro* mutagenesis to change the ATG start codon to ATT preventing gag expression. This mutagenized fragment is 200 base pairs (bp) in length and flanked by Pst I restriction sites. The Pst I-Pst I mutated fragment is purified from the SK^+ plasmid and inserted into the Pst I site of N2 MoMLV 5' LTR in plasmid pUC31 to replace the non-mutated 200 bp fragment. The plasmid pUC31 is derived from pUC19 (Stratagene, La Jolla, CA) in which additional restriction sites Xho I, Bgl II, BssH II and Nco I are inserted between the EcoR I and Sac I sites of the polylinker. This construct is designated pUC31/N2R5gM.

A 1.0 kilobase (Kb) MoMLV 3' LTR EcoR I-EcoR I fragment from N2 is cloned into plasmid SK^+ resulting in a construct designated $N2R3^-$. A 1.0 Kb Cla I-Hind III fragment is purified from this construct.

The Cla I-Cla I dominant selectable marker gene fragment from pAFVXM retroviral vector (Kriegler *et al.*, *Cell 38*:483, 1984; St. Louis *et al.*, *PNAS 85*:3150-3154,1988), comprising a SV40 early promoter driving expression of the neomycin (neo) phosphotransferase gene, is cloned into the SK^+ plasmid. This construct is designated SK^+ SV_2-*neo* A 1.3 Kb Cla I-BstB I gene fragment is purified from the SK^+ SV_2-*neo* plasmid.

KT-3B or KT-1 vectors are constructed by a three part ligation in which the Xho I-Cla I fragment containing the gene of interest and the 1.0 Kb MoMLV 3' LTR Cla I-Hind III fragment are inserted into the Xho I-Hind III site of pUC31/N2R5gM plasmid. This gives a vector designated as having the KT-1 backbone. The 1.3 Kb Cla I-BstB I *neo* gene

fragment from the pAFVXM retroviral vector is then inserted into the Cla I site of this plasmid in the sense orientation to yield a vector designated as having the KT-3B backbone.

Example 2

Oral Administration of Retroviral Vector for Factor VIII

A. Construction of Full-Length and B Domain Deleted Factor VIII cDNA RETROVECTOR™

i. Production of Plasmid Vectors Encoding Full-Length Factor VIII

The following is a description of the construction of several retroviral vectors encoding a full-length factor VIII cDNA. Due to the packaging constraints of retroviral vectors and because selection for transduced cells is not a requirement for therapy, a retroviral backbone, *e.g.*, KT-1, lacking a selectable marker gene is employed.

A gene encoding full length factor VIII can be obtained from a variety of sources. Here, the full length gene is obtained from the plasmid pCIS-F8 (EP 0 260 148 A2, published March 3, 1993), which contains a full length factor VIII cDNA whose expression is under the control of a CMV major immediate-early (CMV MIE) promoter and enhancer. The factor VIII cDNA contains approximately 80 bp of 5' untranslated sequence from the factor VIII gene and a 3' untranslated region of about 500 bp. In addition, between the CMV promoter and the factor VIII sequence lies a CMV intron sequence, or "cis" element. The cis element, spanning 280 bp, comprises a splice donor site from CMV major immediate-early promoter about 140 bp upstream of a splice acceptor from an immunoglobulin gene , with the intervening region being supplied by an Ig variable region intron.

ii. Construction of a Plasmid Encoding Retroviral Vector ND-5.

A plasmid vector encoding a truncation of about 80% (approximately 370 bp) of the 3' untranslated region of the factor VIII cDNA, designated pND-5, is constructed in a pKT-1 vector as follows: As described below for pJW-2, the pKT-1 vector employed has its Xho I restriction site replaced by that for Not I. The factor VIII insert is generated by digesting pCIS-F8 with Cla I and Xba I, the latter enzyme cutting 5' of the factor VIII stop codon. The approximately 7 kb fragment containing all but the 3' coding region of the factor VIII gene is then purified. pCIS-F8 is also digested with Xba I and Pst I to release a 121 bp

fragment containing the gene's termination codon. This fragment is also purified and then ligated in a three way ligation with the larger fragment encoding the rest of the factor VIII gene and Cla I/Pst I restricted BLUESCRIPT® KS$^+$ plasmid (Stratagene, *supra*) to produce pND-2.

The unique Sma I site in pND-2 is then changed to a Cla I site by ligating Cla I linkers (New England Biolabs, Beverly, MA) under dilute conditions to the blunt ends created by a Sma I digest. After recircularization and ligation, plasmids containing two Cla I sites are identified and designated pND-3.

The factor VIII sequence in pND-3, bounded by Cla I sites and containing the full length gene with a truncation of much of the 3' untranslated region, is cloned as follows into a plasmid backbone derived from a Not I/Cla I digest of pJW-1 [a pKT-1 derivative by cutting at the Xho I site, blunting with Klenow, and inserting a Not I linker (New England Biolabs)], which yields a 5.2 kb Not I/Cla I fragment. pCIS-F8 is cleaved with Eag I and Eco RV and the resulting fragment of about 4.2 kb, encoding the 5' portion of the full length factor VIII gene, is isolated. pND-3 is digested with Eco RV and Cla I and a 3.1 kb fragment is isolated. The two fragments containing portions of the factor VIII gene are then ligated into the Not I/Cla I digested vector backbone to produce a plasmid designated pND-5.

The factor VIII sequence in pND-3, bounded by Cla I sites and containing the full length gene with a truncation of much of the 3' untranslated region, is cloned as follows into a plasmid backbone derived from a Not I/Cla I digest of pJW-1 [a pKT-1 derivative by cutting at the Xho I site, blunting with Klenow, and inserting a Not I linker (New England Biolabs)], which yields a 5.2 kb Not I/Cla I fragment. pCIS-F8 is cleaved with Eag I and Eco RV and the resulting fragment of about 4.2 kb, encoding the 5' portion of the full length factor VIII gene, is isolated. pND-3 is digested with Eco RV and Cla I and a 3.1 kb fragment is isolated. The two fragments containing portions of the factor VIII gene are then ligated into the Not I/Cla I digested vector backbone to produce pND-5.

As those in the art will appreciate, after construction of plasmids encoding retroviral vectors such as are described above, such plasmids can then be used in the production of various cell lines from which infectious recombinant retroviruses can be produced. The production of such cell lines is described in the following example.

These constructs are used to make infectious vector particle as described in Examples 2Ca-2Cc below.

iii. Construction of a Plasmid Encoding Retroviral Vector JW-2.

A plasmid, pJW-2, encoding a retroviral vector for expressing full length factor VIII is constructed using the KT-1 backbone from pKT-1. To facilitate directional

cloning of the factor VIII cDNA insert into pKT-1, the unique Xho I site is converted to a Not I site by site directed mutagenesis. The resultant plasmid vector is then opened with Not I and Cla I. pCIS-F8 is digested to completion with Cla I and Eag I, for which there are two sites, to release the fragment encoding full length factor VIII. This fragment is then ligated into the Not I/Cla I restricted vector to generate pJW-2.

iv Construction of the B-Domain Deleted Vector

The precursor DNA for the B-deleted FVIII is obtained from Miles Laboratory. This expression vector is designated p25D and has the exact backbone as pCISF8 above. The Hpa I site at the 3' of the FVIII8 cDNA in p25D is modified to Cla-I by oligolinkers. An Acc I to Cla I fragment is clipped out from the modified p25D plasmid. This fragment spans the B-domain deletion and includes the entire 3' two-thirds of the cDNA. An Acc I to Cla I fragment is removed from the Retrovector™ JW-2 above, and replaced with the modified B-domain deleted fragment just described. This is designated B-del-1

B. i. Assay of Utility of KT-ND5 Vector Expression by Transient Packaging and Transduction of Murine Cells

Cell lines, L33, (Dennert, USC Comprehensive Cancer Center, Los Angeles, CA, Patek, *et. al., Int. J. of Cancer 24*:624-628, 1979), BC10ME (Patek, *et al.*, Cell Immuno 72:113, 1982, ATCC# TIB85), L33env, and BCenv (L33*env* and BC*env* express HIV-1 IIIB*env*, Warner et al, AIDS Res. and Human Retrovirus 7:645, 1991), transduced with the KT-ND5 vector, carrying the amphotropic or VSVG envelope protein are examined for the expression of factor VIII. Non-transduced cells are also analyzed for factor VIII expression and compared with KT-ND5 transduced cells to determine the effect of transduction on protein expression.

Murine cell lines, L33-KT-ND5, L33*env*-KT-ND5, L33*env*, L33, BC10ME, BC10ME-KT-ND5, BC*env* , and BC*env*-KT-ND5, are tested for expression of the KT-ND5 molecule. Cells are grown to subconfluent density and the supernatant is removed following centrifugation at 200 xg. The samples are diluted and assayed by the COATEST® Factor VIII assay (KabiVitrum Diagnostica, Molndal, Sweden).

The assay is performed as follows: 100 µl of culture media sample is mixed with 200 µl of working buffer provided in the kit. The mixture is incubated at 37°C for 4 - 5 min., after which 100 µL of a 0.025 M $CaCl_2$ stock solution is added, followed by a 5 min. 37°C incubation. 200 µL of the chromogenic reagent (20 mg S-2222, 335 µg synthetic thrombin inhibitor, I-2581, in 10 mL) is then mixed in. After a 5 min. incubation at 37°C,

100 µL of 20% acetic acid or 2% citric acid is added to stop the reaction. Absorbance is then measured against a blank comprising 50 mM Tris, pH 7.3, and 0.2% bovine serum albumin (BSA). A standard curve based on dilutions of normal human plasma (1.0 IU factor VIII/mL) is used and the assays should be performed in plastic tubes. Serum levels of factor VIII in non-hemophilic patients are in the range of 200 ng/mL.

When this assay is used for patient samples, 9 volumes of blood is mixed with one volume of 0.1 M sodium citrate, pH 7.5, and centrifuged at 2,000 x g for 5 - 20 min. at 20 - 25°C to pellet cells. Due to heat lability of factor VIII, plasma samples should be tested within 30 min. of isolation or stored immediately at -70°C, although as much as 20% of factor VIII activity may be lost during freezing and thawing.

ii. Assay of Utility of KT-ND5 Vector Expression by Transient Packaging and Transduction of Human Cells

Cell lines transduced with KT-ND5 are examined for expression of factor VIII. Non-transduced cells are analyzed to compare with KT-ND5 transduced cells and determine the effect that transduction has on expression.

Two human cell lines, JY and JY-KT-ND5 are tested for expression of KT-ND5. Suspension cells grown to 10^6 cells/ml are removed from culture flasks by pipette and pelleted by centrifugation at 200 xg. The supernatant is removed, diluted, and assayed by the COATEST® Factor VIII assay as described above in Example 2Bi.

C. Transient Transfection and Transduction of Packaging Cell Lines HX and DA with the Vector Construct KT-ND5

a. Plasmid DNA Transfection

The packaging cell line, HX (WO92/05266), are seeded at 5.0 x 10^5 cells on a 10 cm tissue culture dish on day 1 with Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS). On day 2, the media is replaced with 5.0 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA co-precipitation is performed by mixing 40.0 µl 2.5 M $CaCl_2$, 10 µg plasmid DNA, and deionized H_2O to a total volume of 400 µl. Four hundred microliters of the DNA-$CaCl_2$ solution is added dropwise with constant agitation to 400 µl precipitation buffer (50 mM HEPES-NaOH, pH 7.1; 0.25 M NaCl and 1.5 mM Na_2HPO_4-NaH_2PO_4). This mixture is incubated at room temperature for 10 minutes. The resultant fine precipitate is added to a culture dish of cells. The cells are incubated with the DNA precipitate overnight at 37°C. On day 3, the media is

aspirated and fresh media is added. The supernatant is removed on day 4, passed through a 0.45 µl filter, and stored at -80°C.

Alternatively, 29 2 3 cells (WO 92/05266) (these are 293 cells expressing gag and pol) are transfected with the vector DNA and the plasmid pMLP-VSVG (or other VSVG encoding plasmids) to yield VSVG psuedotyped vector particles that are harvested and stored as described above.

b. Packaging Cell Line Transduction and Generation of Producer Lines

DA (an amphotropic cell line derived from a D17 cell line ATCC No. 183, WO 92/05266) cells are seeded at 5.0 x 10^5 cells/10 cm tissue culture dish in 10 ml DMEM and 10% FBS, 4 µg/ml polybrene (Sigma, St. Louis, MO) on day 1. On day 2, 3.0 ml, 1.0 ml and 0.2 ml of the freshly collected virus-containing HX media is added to the cells. The cells are incubated with the virus overnight at 37°C. On day 3, the media is removed and 1.0 ml DMEM, 10% FBS with 800 µg/ml G418 is added to the plate. Only cells that have been transduced with the vector and contain the neomycin selectable marker will survive. A G418 resistant pool is generated over a period of a week. The pool of cells is dilution cloned by removing the cells from the plate and counting the cell suspension, diluting the cells suspension down to 10 cells/ml and adding 0.1 ml to each well (1 cell/well) of a 96 well plate (Corning, Corning, NY). Cells are incubated for 14 days at 37°C, 10% CO_2. Twenty-four clones are selected and expanded up to 24 well plates, 6 well plates then 10 cm plates at which time the clones are assayed for expression and the supernatants are collected and assayed for viral titer.

The titer of the individual clones is determined by infection of HT1080 cells, (ATCC No. CCL 121). On day 1, 5.0 x 10^5 HT1080 cells are plated on each well of a 6 well microtiter plate in 3.0 ml DMEM, 10% FBS and 4 µg/ml polybrene. On day 2, the supernatant from each clone is serially diluted 10 fold and used to infect the HT1080 cells in 1.0 ml aliquots. The media is replaced with fresh DMEM, 10% FBS media, and the cells incubated with the vector overnight at 37°C, 10% CO_2. On day 3, selection of transduced cells is performed by replacing the media with fresh DMEM, 10% FBS media containing 800 µg/ml G418. Cells are incubated at 37°C, 10% CO_2 for 14 days at which time G418 resistant colonies are scored at each dilution to determine the viral titer of each clone as colony forming units(cfu)/ml.

Using these procedures, cell lines are derived that produce greater than or equal to 10^6 cfu/ml in culture.

The packaging cell line HX is transduced with vector generated from the DA vector producing cell line in the same manner as described for transduction of the DA cells from HX supernatant.

Transduction of the DA or HX cells with vectors lacking a *neo* selectable marker (Example 1) was performed as described above. However, instead of adding G418 to the cells on day 3, the cells are cloned by limiting dilution. Titer is analyzed as described above.

c. Alternative Method of Generation of Producer Cell Line via One Packaging Cell Line

In some situations it may be desirable to avoid using more than one cell line in the process of generating producer lines. In this case, DA cells are seeded at 5.0×10^5 cells on a 10 cm tissue culture dish on day 1 with DMEM and 10% irradiated (2.5 megarads minimum) FBS. On day 2, the media is replaced with 5.0 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA coprecipitation is performed by mixing 60 µl 2.0 M $CaCl_2$, 10 µg MLP-G plasmid, 10 µg KT-ND5 retroviral vector plasmid, and deionized water to a volume of 400 µl. Four hundred microliters of the DNA-$CaCl_2$ solution is added dropwise with constant agitation to 400 µl 2X precipitation buffer (50 mM HEPES-NaOH, pH 7.1, 0.25 M NaCl and 1.5 mM Na_2HPO_4-NaH_2PO_4). This mixture is incubated at room temperature for 10 minutes. The resultant fine precipitate is added to a culture dish of DA cells plated the previous day. The cells are incubated with the DNA precipitate overnight at 37°C. On day 3, the medium is removed and fresh medium is added. The supernatant containing G-pseudotyped virus is removed on day 4, passed through a 0.45 µl filter and used to infect the DA packaging cell.

DA cells are seeded at 5.0×10^5 cells on a 10 cm tissue culture dish in 10 ml DMEM and 10% FBS, 4 mg/ml polybrene (Sigma, St. Louis, MO) on day 1. On day 2, 2.0 ml, 1.0 ml or 0.5 ml of the freshly collected and filtered G-pseudotyped virus containing supernatant is added to the cells. The cells are incubated with the virus overnight at 37°C. On day 3 the medium is removed and 10 ml DMEM, 10% irradiated FBS with 800 µg/ml G418 is added to the plate. Only cells that have been transduced with the vector and contain the *neo* selectable marker will survive. A G418 resistant pool is generated over the period of 1-2 weeks. The pool is tested for expression and then dilution cloned by removing the cells from the plate, counting the cell suspension, diluting the cell suspension down to 10 cells/ml and adding 0.1 ml to each well (1 cell/well) of a 96-well plate. Cells are incubated for 2 weeks at 37°C, 10% CO_2 Twenty-four clones are selected and expanded up to 24-well plates, then 6-well plates, and finally 10 cm plates, at which time the clones are assayed for expression and the supernatants are collected and assayed for viral titer as described above.

D. Detection of Replication Competent Retroviruses (RCR)

i. The Extended S^+L^- Assay

The extended S^+L^- assay determines whether replication competent, infectious virus is present in the supernatant of the cell line of interest. The assay is based on the empirical observation that infectious retroviruses generate foci on the indicator cell line $MiCl_1$ (ATCC No. CCL 64.1). The $MiCl_1$ cell line is derived from the Mv1Lu mink cell line (ATCC No. CCL 64) by transduction with Murine Sarcoma Virus (MSV). It is a non-producer, non-transformed, revertant clone containing a replication defective murine sarcoma provirus, S^+, but not a replication competent murine leukemia provirus, L^-. Infection of $MiCl_1$ cells with replication competent retrovirus "activates" the MSV genome to trigger "transformation" which results in foci formation.

Supernatant is removed from the cell line to be tested for presence of replication competent retrovirus and passed through a 0.45 μ filter to remove any cells. On day 1, Mv1Lu cells are seeded at 1.0×10^5 cells per well (one well per sample to be tested) of a 6 well plate in 2 ml DMEM, 10% FBS and 8 μg/ml polybrene. Mv1Lu cells are plated in the same manner for positive and negative controls on separate 6 well plates. The cells are incubated overnight at 37°C, 10% CO_2. On day 2, 1.0 ml of test supernatant is added to the Mv1Lu cells. The negative control plates are incubated with 1.0 ml of media. The positive control consists of three dilutions (200 focus forming units (ffu), 20 ffu and 2 ffu each in 1.0 ml media) of MA virus (referred to as pAM in Miller *et al., Molec. and Cell Biol. 5*:431, 1985) which is added to the cells in the positive control wells. The cells are incubated overnight. On day 3, the media is aspirated and 3.0 ml of fresh DMEM and 10% FBS is added to the cells. The cells are allowed to grow to confluency and are split 1:10 on day 6 and day 10, amplifying any replication competent retrovirus. On day 13, the media on the Mv1Lu cells is aspirated and 2.0 ml DMEM and 10% FBS is added to the cells. In addition, the $MiCl_1$ cells are seeded at 1.0×10^5 cells per well in 2.0 ml DMEM, 10% FBS and 8 μg/ml polybrene. On day 14, the supernatant from the Mv1Lu cells is transferred to the corresponding well of the $MiCl_1$ cells and incubated overnight at 37°C, 10% CO_2. On day 15, the media is aspirated and 3.0 ml of fresh DMEM and 10% FBS is added to the cells. On day 21, the cells are examined for focus formation (appearing as clustered, refractile cells that overgrow the monolayer and remain attached) on the monolayer of cells. The test article is determined to be contaminated with replication competent retrovirus if foci appear on the $MiCl_1$ cells. Using these procedures, it can be shown that the HBV core producer cell lines are not contaminated with replication competent retroviruses.

ii. Cocultivation of Producer Lines and MdH Marker Rescue Assay

As an alternate method to test for the presence of RCR in a vector-producing cell line, producer cells are cocultivated with an equivalent number of *Mus dunni* (NIH NIAID Bethesda, MD) cells. Small scale cocultivations are performed by mixing of 5.0 x 10^5 *Mus dunni* cells with 5.0 x 10^5 producer cells and seeding the mixture into 10 cm plates (10 ml standard culture media/plate, 4 µg/ml polybrene) at day 0. Every 3-4 days the cultures are split at a 1:10 ratio and 5.0 x 10^5 *Mus dunni* cells are added to each culture plate to effectively dilute out the producer cell line and provide maximum amplification of RCR. On day 14, culture supernatants are harvested, passed through a 0.45 µ cellulose-acetate filter, and tested in the MdH marker rescue assay. Large scale cocultivations are performed by seeding a mixture of 1.0 x 10^8 *Mus dunni* cells and 1.0 x 10^8 producer cells into a total of twenty T-150 flasks (30 ml standard culture media/flask, 4 µg/ml polybrene). Cultures are split at a ratio of 1:10 on days 3, 6, and 13 and at a ratio of 1:20 on day 9. On day 15, the final supernatants are harvested, filtered and a portion of each is tested in the MdH marker rescue assay.

The MdH marker rescue cell line is cloned from a pool of *Mus dunni* cells transduced with LHL, a retroviral vector encoding the hygromycin B resistance gene (Palmer *et al., PNAS 84*: 1055-1059, 1987). The retroviral vector can be rescued from MdH cells upon infection of the cells with RCR. One ml of test sample is added to a well of a 6-well plate containing 10^5 MdH cells in 2 ml standard culture medium (DMEM with 10% FBS, 1% 200 mM L-glutamine, 1% non-essential amino acids) containing 4 µg/ml polybrene. Media is replaced after 24 hours with standard culture medium without polybrene. Two days later, the entire volume of MdH culture supernatant is passed through a 0.45 µ cellulose-acetate filter and transferred to a well of a 6-well plate containing 5.0 x 10^4 *Mus dunni* target cells in 2 ml standard culture medium containing polybrene. After 24 hours, supernatants are replaced with standard culture media containing 250 µg/ml of hygromycin B and subsequently replaced on days 2 and 5 with media containing 200 µg/ml of hygromycin B. Colonies resistant to hygromycin B appear and are visualized on day 9 post-selection, by staining with 0.2% Coomassie blue.

F. Transfer of Expression Assay on HT1080 Human Fibroblasts with a FVIII Expressing Retroviral Vector

On day one, HT1080 cells are set up at 2 x 10^4 cells per well in six well tissue culture plates containing 2 mls standard growth media (DME + 10% FBS). On day two, ND-5 FVIII retroviral vector particles from a confluent vector producing cell line are harvested as a HX-ND-5 clone. They are filtered through .45 µm syringe filters prior to testing the supernatants. (Alternatively the filtered media supernatants may be frozen at 80 in

aliquots for later use.) Polybrene is added to each well such that the final concentration is 8 μg per ml. Thirty minutes later, either diluted or undiluted retroviral vector supernatant is added to duplicate wells. Typical volumes and dilutions are 0.5 ml per well and four or more 1:3 serial dilutions in growth media. As a control, two wells are transduced with the same volume of growth media only. On day three, the wells are refeed with 2 mls of fresh media and the cells allowed to reach confluence, which may typically be about day four or five. On this day, the cells are again refeed with one ml per well fresh growth media. Twenty four hours later the media is harvested and filtered as above.

G. Expression of Transduced Vector for FVIII

The expression of vector transduced human cells for FVIII is detected by the COATEST^R assay as described above in Example 2Bi. Activity is assayed relative to supernatant from the control wells by counting the cells per well from the two control wells and normalizing FVIII expression data per 1x 10^6 cells per 24 hours.

H. Administration of Vector Construct

i. Animal Administration Protocol

The intestinal epithelium is an attractive site for gene delivery due to its rapidly proliferating tissue mass and the known location of stem cells in the crypts of Lieberkuhn. The deep location of the stem cells in the crypts and the protective role of the mucus gel layer, makes the retrovirus relatively inaccessible to the tissue cells. However, the accessibility of the retroviral vector to these stem cells can be improved in animal models by the *in vivo* mucus removal method of Sandberg, J., *et al.*,(*Human Gene Therapy 5*:3232-329, 1994).

Male Sprague-Dawley rats obtained from Charles River Breeding Laboratories (Portage, MD.) are anesthetized and the cecum is identified upon opening the peritoneal cavity. A 3 cm ileal segment is isolated from the last Peyer's patch in the terminal ileum and ligated at each end. A plastic catheter attached to a syringe is inserted into the segment and two milliliters of the mucolytic agents dithiothreitol and N-acetyl-cysteine is instilled under mild pressure for two minutes, then removed. This procedure is repeated once again before filling the segment with 0.2 to 2.0 ml of retroviral vector particles at, 10^6, 10^7, 10^8, 10^9, 10^{10} (10^6 to 10^{10}) cfu/ml. The ligatures are removed 1 to 4 hours later and the abdominal cavity is sutured. Control animals are instilled with formulation buffer only.

Blood is collected from the tail vein and assayed for factor VIII production by a sandwich ELISA specific for human factor VIII (according to the modified procedure of

Zatloukal, K., *et al., PNAS 91*:5148-5152, 1994). The ELISA is based on two monoclonal antibodies directed against human factor VIII (ESH 4 and ESH 8: American Diagnostica). ESH 4 (25 μg/ml in 1.0 M $NaHCO_3$/0.5 M NaCl, pH 9.0) is coupled to the ELISA plates overnight at 4°C, washed with 0.1% Tween 20 in PBS, and blocked with 1% BSA in PBS. The samples are applied in 0.05 M Tris-HCl/1 M NaCl/2% BSA, pH 7.5, over 4 hr at room temperature, the plates are washed, and ESH 8 (2.5 μg/ml in 0.05 M Tris-HCl/1 M NaCl/2% BSA, pH 7.5,) which has been biotinylated with *N*- hydroxysuccinimidobiotin (Pierce, Rockford, IL.) is added for 2 hr at room temperature. The color reaction is performed with peroxidase-conjugated streptavidin (Boehringer Mannheim, Indianapolis, IN.) and o-phenylenediamine dihydrochloride as substrate. The human factor VIII:c standard (from the National Institute for Biological Standards and Control, Hertfordshire, U.K.) and normal rat plasma are used as references.

ii. Human Administration Protocol

Lyophilized retroviral vector containing the gene for Factor VIII expression is formulated into an enteric coated tablet or gel capsule according to known methods in the art. These are described in the following patents: US 4,853,230, EP 225,189, AU 9,224,296, AU 9,230,801, and WO 92144,52.

The capsule is preferably administered orally to be targeted to the jejunum. The encapsidation dose is 10^6, 10^7, 10^8, 10^9, 10^{10}, or 10^{11} (10^6 to 10^{11})cfu or equivalent units. At 1 to 4 days following oral administration of the retroviral vector, expression of Factor VIII is measured in the plasma and blood by the COATEST^R Factor VIII assay as described in Example 2Bi.

Example 3

Intravesical Administration of Retroviral Vectors Expressing TK

A. Construction of TK Vector Constructs

i. Construction of plasmids containing vector LTR sequences

All of the following retroviral vectors are based on the N2 vector (Keller et al., *Nature 318*:149-154, 1985). Briefly, 5' and 3' Eco RI LTR fragments (2.8 and 1.0 Kb, respectively) (Armentano, *J. Vir. 61*:1647, 1987; Eglitis, *Science 230*:1395, 1985) are

initially subcloned into the Eco RI site of plasmids SK^+ (Stratagene, San Diego, CA) and pUC31. pUC31 is a modification of pUC19 (Stratagene, San Diego, CA) carrying additional restriction sites (Xho I, Bgl II, BssH II, and Nco I) between the Eco RI and Sac I sites of the polylinker. Plasmid N2R3+/- is thereby created from ligation of the SK^+ plasmid with the 1.0 Kb 3' LTR fragment. The plasmids p31N2R5+/- and p31N2R3+/- are constructed from the ligation of pUC31 with the 2.8 Kb 5' LTR and packaging signal (Y) or the 1.0 Kb 3' LTR fragment, respectively. In each case N2 refers to the vector source, R refers to the fact that the fragment is an Eco RI fragment, 5 and 3 refer to 5' or 3' LTRs, and + or - refers to the orientation of the insert (see Figures 1-6 for examples of LTR subclones).

In one case, a 1.2 Kb Cla I/Eco RI 5' LTR and W fragment from N2 is subcloned into the same sites of an SK^+ vector. This vector is designated pN2CR5. In another case, the 5' LTR containing a 6 bp deletion of the splice donor sequence (Yee *et al.*, Cold Spring Harbor, Quantitative Biology, 51:1021, 1986) is subcloned as a 1.8 Kb Eco RI fragment into pUC31. This vector is designated p31N25D[+], Figure 6.

ii. Construction of plasmids containing HSVTK

The coding region and transcriptional termination signals of HSV-1 thymidine kinase gene (HSVTK) are isolated as a 1.8 Kb Bgl II/Pvu II fragment from plasmid 322TK (3.5 kb Bam HI fragment of HSV-1 (McKnight *et al.*) cloned into Bam HI of pBR322 (ATCC No. 31344)) and cloned into Bgl II/Sma I-digested pUC31. This construct is designated pUCTK. For constructs which require deletion of the terminator signals, pUCTK is digested with Sma I and Bam HI and the 0.3 Kb fragment containing the $(A)_n$ signal is removed. The remaining coding sequences and sticky-end Bam HI overhang are reconstituted with a double-stranded oligonucleotide made from the following oligomers:

5' GAG AGA TGG GGG AGG CTA ACT GAG 3' (SEQUENCE ID. NO. 1)

5' GAT CCT CAG TTA GCC TCC CCC ATC TCT C 3' (SEQUENCE ID. NO. 2)

The resulting construct is designated pTKD A, Figure 7.

For diagnostic purposes, the oligonucleotides are designed to destroy the Sma I site while maintaining the Ava I site without changing the translated protein.

The plasmid pPrTKDA (Figure 8), which contains the HSVTK promoter and coding sequence (lacking an $(A)_n$ signal), is constructed as follows.

1. pTKD A is linearized with Bgl II treated with alkaline phosphatase, and gel purified.

2. A 0.8 Kg fragment contained the HSVTK transcriptional promoter is isolated as a Bam HI/Bgl II fragment from p322TK.

3. Products from (1) and (2) are ligated, transformed into bacteria, and positive clones are screened for the proper orientation of the promoter region. A resultant clone is designated pPrTKDA (Figure 8).

iii. Construction of retroviral provectors expressing HSVTK from a constitutive promoter

The retroviral provectors pTK-1 and pTK-3 are constructed essentially as described below.

1. The 5 Kb Xho I/Hind III 5' LTR and plasmid sequences are isolated from p31N2R5(+) (Figure 1).

2. HSVTK coding sequences lacking transcriptional termination sequences are isolated as a 1.2 Kb Xho I/Bam HI fragment from pTKDA (Figure 2).

3. 3' LTR sequences are isolated as a 1.0 Kb Bam HI/Hind III fragment from pN2R3(-) (Figure 2).

4. The fragments from steps 1-3 are mixed, ligated, transformed into bacteria, and individual clones identified by restriction enzyme analysis. The construct is designated TK-1 (Figure 9).

5. pTK-3 is constructed by linearizing TK-1 with Bam HI, filling in the 5' overhang and blunt-end ligating a 5'-filled Cla I/Cla I fragment containing the bacterial lac UV5 promoter, SV40 early promoter, plus Tn5 neo^r gene obtained from pAFVXM retroviral vector (Krieger *et al.*, *Cell 39*:483, 1984; St. Louis *et al.*, *PNAS 85*:3150, 1988). Kanamycin-resistant clones are isolated and individual clones are screened for the proper orientation by restriction enzyme analysis (Figure 9).

These constructs were used to generate infectious recombinant vector particles in conjunction with a packaging cell line, such as DA as described above.

B. Determination of the Effect of Ganciclovir on Mouse Colon Carcinoma Cells With or Without TK-3 Vector

An experiment was performed to determine whether or not ganciclovir had an effect on CT26 cells (colon tumor 26, Brattain, Baylor College of Medicine, Houston, TX) that were transduced with DA/TK-3. CT26 cells are transduced with G-pseudotyped TK-3 vector. Twenty-four hours after adding the viral supernatant, the CT26 cells are placed under G-418 selection (450 lg/ml). After 10 days incubation, a G-418 selected pool is obtained and designated CT26TK Neo. CT26 TK Neo cells were seeded into six 10 cm^2 plates at a density of 2.5 X 10^6 per plate. As controls, each of two other cell types, CT26 and CT26 beta-*gal* (this cell line was transduced with a virus carrying the reporter gene β-galactosidase from *E. coli.*), were also seeded into six 10 cm^2 plates as controls. Five plates

of each cell type were treated twice per day for four consecutive days with medium containing ganciclovir concentrations of 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml and 6.25 µg/ml. One plate of each cell type was left untreated. Afterwards, the cells were removed from each dish using trypsin-EDTA, resuspended in DMEM with 10% FBS and counted. The data in Figure 10 shows that even the lowest dose of ganciclovir had a dramatic cytotoxic effect on the CT26 TKneo cells. This dose of ganciclovir (6.25 µg/ml) or even the next higher dose (12.5 µg/ml) did not have an effect on either the CT26 or CT26 beta-*gal* cells. However, beginning at a ganciclovir dose of 25 µg/ml, a dose-dependent decrease in cell growth could be seen, although CT26 TK Neo cells were always more sensitive to the drug.

C. Determination of a Ganciclovir Dose For the Treatment of Mice Injected with CT26 TK Neo Cells

In order to test whether *in vivo* transduction of a murine tumor could be used to treat the disease, an experiment was performed to determine the optimal concentration of ganciclovir necessary to eliminate a tumor that was transduced and selected *in vitro* to assure 100% transduction. Twelve groups of 3 mice each are injected with 2×10^5 CT26TK Neo cells. Six groups of mice are injected with these cells intraperitoneally (I.P.) and six groups of mice are injected subcutaneously (S.C.). Two other groups of 3 mice each are injected with 2×10^5 unmodified CT26 cells (as a control) either I.P. or S.C..

Ten days after the injection of the CT26 or CT26TK Neo cells into these groups of mice, several concentrations of ganciclovir treatment are initiated. Each dose regimen consists of 2 daily AM and PM I.P. injections of ganciclovir. The experiment is summarized in Table A below.

TABLE A

Group	Innoculum	Injection Route	Concentration of Ganciclovir (Mg/Kg)
1	CT26 I.P.	0	
2	CT26 TKneo	I.P.	0
3	CT26 TKneo	I.P.	15.63
4	CT26 TKneo	I.P.	31.25
5	CT26 TKneo	I.P.	62.5
6	CT26 TKneo	I.P.	125.0
7	CT26 TKneo	I.P.	250.0
8	CT26 TKneo	I.P.	500.0
9	CT26	Subq.	0
10	CT26 TKneo	Subq	0
11	CT26 TKneo	Subq.	15.63
12	CT26 TKneo	Subq.	31.25
13	CT26 TKneo	Subq.	62.5
14	CT26 TKneo	Subq.	125.0
15	CT26 TKneo	Subq.	250.0
16	CT26 TKneo	Subq.	500.0

After 5 days, all of the mice in the 125 mg/Kg, 250 mg/Kg and 500 mg/Kg treated groups were dead due to the toxic effects of ganciclovir. Mice in the 15.63 mg/Kg, 31.25 mg/Kg and 62.5 mg/Kg treated groups were treated for an additional 7 days and were able to tolerate the treatment. Tumor measurements were made for 23 days Figure 11). CT26TK Neo grew only slightly slower than unmodified CT26 in the absence of ganciclovir. Complete tumor regression was seen in the groups of mice treated with the 62.5 mg/Kg regimen. Partial tumor regression was seen in the 31.25 mg/Kg treated groups. Little or no effect was seen in the 15.63 mg/Kg treated groups as compared to the 2 untreated control groups. Even though there was some toxicity observed in the 62.5 mg/Kg groups, it was not life threatening and reversible upon the discontinuation of the treatments so this concentration was used for future studies (Figure 11). After 24 days, the I.P. injected animals were sacrificed and evaluated. As seen in Figures 12 and 13 the optimal concentration for anti-tumor effect was similar whether the tumor was grown I.P. or S.C..

D. Comparison of Cytotoxicity on CT26 and CT26TK Neo In Vivo Tumor Growth

In order to determine whether ganciclovir has an effect on the growth of unmodified CT26 tumor cells *in vivo*, 2 groups of 7 mice are injected S.C. with 2 X 10^5 unmodified CT26 cells and 2 groups of 7 mice are injected S.C. with 2 X 10^5 CT26TK Neo cells. Seven days after tumor implantation, one group of CT26 injected mice and one group of CT26TK Neo injected mice are placed on a twice daily (AM and PM) regimen of I.P.

ganciclovir at 62.5 mg/Kg. These mice are treated for 12 days or until the CT26TK Neo injected animals have no detectable tumor burden. Tumor growth is monitored over a three week period. Mice injected with CT26 and treated with ganciclovir had tumors that were somewhat smaller than untreated mice injected with CT26, indicating a small HSVTK-independent inhibition of tumor growth (Figure 14). However, a dramatic decrease in tumor burden was observed if, and only if, CT26 TKneo containing mice were treated with ganciclovir (Figure 14).

E. Determination of the Effect of Ganciclovir on AY-27 Rat Carcinoma Cells With or Without the TK-3 Vector

An experiment is performed to determine whether or not ganciclovir has an effect on AY-27 cells that are transduced with DA/TK-3. AY-27 cells are rat carcinoma cells which have been induced by N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (Selman, S., *et al., J. Urol. 136*:141, 1986). The AY-27 cells are transduced with G-pseudotyped TK-3 vector. Twenty-four hours after adding the viral supernatant, the AY-27 cells are placed under G-418 selection (450 μg/ml). After 10 days incubation, a G-418 selected pool is obtained and designated AY-27TK Neo. AY-27TK Neo cells are seeded into six 10 cm^2 plates at a density of 2.5 X 10^6 per plate. As controls, each of two other cell types, AY-27 and AY-27beta-*gal* (this cell line is transduced with a virus carrying the reporter gene β-galactosidase from *E. coli.*), are also seeded into six 10 cm^2 plates as controls. Five plates of each cell type are treated twice per day for four consecutive days with medium containing ganciclovir concentrations of 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml and 6.25 μg/ml. One plate of each cell type is left untreated. Afterwards, the cells are removed from each dish using trypsin-EDTA, resuspended in DMEM with 10% FBS and counted. The data generated can be used to determine the concentration which has the most cytotoxic effect o the AY-27, AY-27 beta-*gal*, or AY-27 TK Neo cells.

F. Determination of a Ganciclovir Dose For the Treatment of Rats Injected with AY-27 TK Neo Cells

In order to test whether *in vivo* transduction of a murine tumor could be used to treat the disease, an experiment was performed to determine the optimal concentration of ganciclovir necessary to eliminate a tumor that was transduced and selected *in vitro* to assure 100% transduction. Fourteen groups of 3 rats each are injected with cells. Seven groups of 3 rats each are injected intravesically with 2 X 10^5 unmodified AY-27 cells (as a control), and seven groups with 2 X 10^5 AY-27TK Neo cells.

Ten days after the injection of the AY-27 or AY-27TK Neo cells into these groups of rats, several concentrations of ganciclovir treatment are initiated. Each dose regimen consists of 2 daily AM and PM I.P. injections of ganciclovir. The experiment is summarized in Table B below.

TABLE B

Group	Innoculum	Injection Route	Concentration of Ganciclovir (Mg/Kg)
1	AY-27	intravesically	0
2	AY-27	intravesically	15.63
3	AY-27	intravesically	31.25
4	AY-27	intravesically	62.5
5	AY-27	intravesically	125.0
6	AY-27	intravesically	250.0
7	AY-27	intravesically	500.0
8	AY-27 TKneo	intravesically	0
9	AY-27 TKneo	intravesically	15.63
10	AY-27 TKneo	intravesically	31.25
11	AY-27 TKneo	intravesically	62.5
12	AY-27 TKneo	intravesically	125.0
13	AY-27 TKneo	intravesically	250.0
14	AY-27 TKneo	intravesically	500.0

Rats are treated for 12 days, eliminating those that die due to the higher concentrations of ganciclovir. Tumor measurements are made for 20 days, assessing tumor regression in order to determine an optimal ganciclovir concentration.

G. Administration Protocol

i Rat Administration Protocol

AY-27 rat bladder carcinoma cells are transplanted into the bladders of 20 male Fischer 344 rats (Charles River Breeding Laboratories, Portage, MD). One to fourteen days following transplantation, tumor-bearing rats weighing between 200 to 300 grams are anesthetized, their bladders are surgically exteriorized and evacuated of urine with a 27-gauge needle. Viral vector particles are instilled into the bladders of 5 groups of rats at 10^6 to 10^{10} cfu in 200 to 2,000 µl of formulation buffer. Four to eight µg/ml of polybrene may be added to increase the efficiency of transduction. In order to prevent leakage, the cystotomy is repaired with 7-zero nonabsorbable suture. The virus is allowed to incubate in the presence of the tumor cells for 0.5 to 1.0 hour by keeping the animal anesthetized and

thereby preventing voiding. Five control rats receive 500 µl of formulation buffer only, and effective doses of vector noted.

Alternatively, in a similarly formatted experiment, 200 to 2,000 µl of vector can be instilled directly into the bladder by catheterization through the urethra following urine evacuation and rinsing with saline.

At 24 to 72 hours after vector treatment, the rats are placed on twice daily (AM and PM) injections of I.P. ganciclovir at the previously determined optimum dose (e.g. 62.5 mg/Kg body weight) for 4 to 12 days. Finally, the rats receive a single daily dose of ganciclovir until the end of the experiment (1 to 10 weeks). Whole bladders are removed and tumor growth is measured.

ii Human Administration Protocol

A urinary (Foley) catheter is inserted through the urethra into the bladder and secured in place. The bladder is evacuated of urine and washed with 100 to 500 mls of sterile saline. Retroviral vector particles containing the gene for thymidine kinase expression are instilled through the catheter into the bladder at 10^5 to 10^{11} cfu in 10 to 500 ml of formulation buffer preferably containing 4 to 8 µg/ml of polybrene, or other enhancing excipients. The viral particles are allowed to incubate for 0.25 to 12 hours prior to removal of the catheter. After 1 to 7 days, ganciclovir is administered at 1 to 5mg/Kg I.V. (at a constant rate over 1 hour) every 12 hours for 2 to 21 days. The vector can be readministered multiple times (2 to 20), followed by ganciclovir administration. Due to the frequency of granulocytopenia and thrombocytopenia in patients receiving ganciclovir, it is recommended that neutrophil and platelet counts be performed every two days during the dosing of the drug. Tumor regression is monitored by x-ray and/or biopsy and the treatment repeated if required.

Example 4

Pulmonary Administration of Retroviral Vectors Expressing Factor VIII

A. Construction of Full-Length and B Domain Deleted Factor VIII cDNA Retrovector™

The construction of the full-length and B domain deleted Factor VIII retrovectors are described in Example 2A.

B. Aerosolization of the Retroviral Vector Expressing Factor VIII

The KT-ND5 viral supernatant in formulation buffer, with 4 to 8 μg/ml of polybrene or other transduction enhancing excipient, is nebulized using a DeVilbiss #15 Atomizer (DeVilbiss Health Care Division, Somerset, PA) designed to produce 0.3 to 0.5 μm particles (Rousculp, M. , *Human Gene Therapy* 3:471-477, 1992.) The aerosol produced by this nebulizer uses compressed air in a laminar flow hood. The mist is directed into a polypropylene tube, and the condensed vapor, as well as the control viral supernatant, is resterilized by 0.22 μm filtration. Vectors pass through this filter without any significant loss of functional activity.

C. Administration of Vector Construct

i Rat Administration Protocol

Rats are anesthetized and the trachea is exposed by anterior midline incision. Retroviral vector particles expressing the factor VIII gene product are diluted in 300μl of formulation buffer, with or without 4 to 8 μg/ml of polybrene or other transduction enhancing excipient, at 10^5 to 10^{10} cfu/ml and instilled into the trachea though a small gauge needle. Control animals are administered 300μl of formulation buffer only. The incision is sutured and the rats are allowed to recover. Two to fourteen days following viral instillation, blood is drawn from the tail vein and examined for factor VIII production as described in Example 2Hi.

ii. Human Administration Protocol

The retroviral vector is administered using a DeVilbiss #15 Atomizer (as described above) for 2 to 60 minutes. Total administration is 10^5, 10^6, 10^7, 10^8, 10^9, or 10^{10} (10^5 to 10^{10}) cfu/ml with or without polybrene or other transduction enhancers. Two to fourteen days following administration of the retroviral vector particles, expression of Factor VIII is measured in the blood and plasma by the Coatest^R Factor VIII assay (as described in Example 2Bi) .

Example 5

Transdermal Administration of Retroviral Vectors Expressing TK-3

A. Construction of TK-3 Vector Construct

Construction and verification of the TK-3 vector construct and retroviral vector particles are described in Examples 3A through 3F.

Administration of Vector Construct

i. Animal Administration Protocol

Cottontail rabbit papillomavirus (CRPV) provides an animal model for the highly oncogenic human papillomavirus (HPV). Papillomas can be induced in the cottontail rabbit and virus infection leads to three different outcomes in the rabbit (Lin, Y. et el., *J Virol 67*:382, 1993). First, papillomas appear and persist for the lifetime of the rabbit; second, papillomas spontaneously regress 2 to 3 months after infection; and third, papillomas progress to carcinomas after 8 to 15 months.

In this experiment, twelve cottontail rabbits (E. Johnson, Rago, KA) are injected with the B strain of CRPV (Stevens, J. *et al.*, *J Virol 30*:891, 1979) by intradermal injection as described by Stevens, J., *et al.*, (*J Virol 30*:891, 1979). Four to six months after infection, when papillomas form, the animals are divided into two groups. In the first group, papillomas of 6 animals are injected with 25 to 100 µl of formulation buffer, with or without 4 to 8 µg/ml of polybrene or other transduction enhancing excipient, at 10^5 to 10^{10} cfu/ml though a small gauge needle. In the second group, control animals are administered 25 to 100 µl of formulation buffer only. At 24 to 72 hours after vector treatment, the rabbits are placed on twice daily (AM and PM) injections of I.P. ganciclovir at the previously determined optimum dose (e.g. 62.5 mg/Kg body weight) for 4 to 12 days. Finally, the rabbits receive a single daily dose of ganciclovir until the end of the experiment (1 to 10 weeks). Papilloma regression is visually monitored for 2 to 14 days.

ii Human Administration Protocol

The clinical cutaneous lesions that result from the human papillomavirus (HPV) include common warts, filiform warts, plantar warts, and anogenital warts (reviewed in Cobb, M., *et al. J. Am. Acad. Derm. 22*:547, 1990). In this experiment, patients are divided into two groups. In the first group, 100 to 500 µl of retroviral vector particles at a concentration of 10^6 to 10^{11} cfu/ml in a formulated ointment, preferably containing 4 to 8

μg/ml of polybrene or other enhancing excipients, are applied to the warts using a transdermal delivery system (TDS).

Transdermal delivery systems (TDS) are capable of delivering a drug through intact skin so that it reaches the systemic circulation in sufficient quantity to be therapeutically beneficial. TDS provides a variety of advantages including elimination of gastrointestinal absorption problems and hepatic first pass effect, reduction of dosage and dose intervals, and improved patient compliance. The major components of TDS are a controlled release device composed of polymers, the drug to be administered, excipients, and enhancers, and a fastening system to fix the device to the skin. A number of polymers have been described which include gelatin, gum arabic, paraffin waxes, and cellulose acetate phthalate (Sogibayasi, K., *et al.*, *J. Controlled Release, 29*:177-185, 1994).

The second group receives 100 to 500 μl of vector particle in formulation buffer only. The areas are covered and the viral particles are allowed to incubate for 0.25 to 12 hours prior to removal of the TDS. After 1 to 7 days, ganciclovir is administered at 1 to 5mg/Kg I.V., preferably at a constant rate over 1 hour) every 12 hours for 2 to 21 days. Alternative administration protocols may be used, if approved by the FDA. The vector can be readministered multiple times (2 to 20), followed by ganciclovir administration. Due to the frequency of granulocytopenia and thrombocytopenia in patients receiving ganciclovir, it is recommended that neutrophil and platelet counts be performed every two days during the dosing of the drug. The regression of the wart is visually monitored for 2 to 14 days.

Example 6

Ocular Administration of Retroviral Vector for E3/19K

A. Cloning of E3/19K Gene into KT-3B

i. Isolation and Purification of Adenovirus

The isolation and purification of adenovirus is described by Green *et al.* *(Methods in Enzymology 58*: 425, 1979). Specifically, five liters of Hela cells (3-6.0 x 10^5 cells/ml) are infected with 100-500 plaque forming units (pfu) per ml of adenovirus type 2 (Ad2) virions (ATCC No. VR-846). After incubation at 37°C for 30-40 hours, the cells are placed on ice, harvested by centrifugation at 230 xg for 20 minutes at 4°C, and resuspended in Tris-HCl buffer (pH 8.1). The pellets are mechanically disrupted by sonication and homogenized in trichlorotrifluoroethane prior to centrifugation at 1,000 xg for 10 min. The upper aqueous layer is removed and layered over 10 mls of CsCl (1.43 g/cm^3) and

centrifuged in a SW27 rotor for 1 hour at 20,000 rpm. The opalescent viral band is removed and adjusted to 1.34 g/cm^3 with CsCl and further centrifuged in a Ti 50 rotor for 16-20 hours at 30,000 rpm. The visible viral band in the middle of the gradient is removed and stored at 4°C until purification of adenoviral DNA.

ii. Isolation and Purification of Adenovirus DNA

The adenovirus band is incubated with protease for 1 hour at 37°C to digest proteins. After centrifugation at 7,800 xg for 10 minutes at 4°C, the particles are solubilized in 5% SDS at room temperature for 30 minutes before being extracted with equal volumes of phenol. The upper aqueous phase is removed, re-extracted with phenol, extracted three times with ether, and dialyzed in Tris buffer for 24 hours. The viral Ad2 DNA is precipitated in ethanol, washed in ethanol, and resuspended in Tris-EDTA buffer (pH 8.1). Approximately 0.5 mg of viral Ad2 DNA is isolated from virus produced in 1.0 L of cells.

iii. Isolation of E3/19K Gene

The viral Ad2 DNA is digested with EcoR I and separated by electrophoresis on a 1% agarose gel. The resulting 2.7 Kb Ad2 EcoR I D fragments, located in the Ad2 coordinate region 75.9 to 83.4, containing the E3/19K gene (Herisse *et al.*, *Nucleic Acids Research 8*:2173, 1980, Cladaras *et al.*, *Virology 140*:28, 1985) are eluted by electrophoresis, phenol extracted, ethanol precipitated, and dissolved in Tris-EDTA (pH 8.1).

iv. Cloning of E3/19K Gene into KT-3B

The E3/19K gene is cloned into the EcoR I site of PUC1813. PUC1813 is prepared as essentially described by Kay *et al.* (*Nucleic Acids Research 15*:2778, 1987) and Gray *et al.* (*PNAS 80*:5842, 1983). The E3/19K is retrieved by EcoR I digestion and the isolated fragment is cloned into the EcoR I site of phosphatase-treated pSP73 plasmid. This construct is designated SP-E3/19K. The orientation of the SP-E3/19K cDNA is verified by using appropriate restriction enzyme digestion and DNA sequencing. In the sense orientation, the 5' end of the cDNA is adjacent to the Xho I site of the pSP73 polylinker and the 3' end adjacent to the Cla I site. The Xho I-Cla I fragment containing the E3/19K cDNA in either sense or antisense orientation is retrieved from the SP-E3/19K construct and cloned into the Xho I-Cla I site of the KT-3BB retroviral. This construct is designated KT-3B/E3/19K.

B. Cloning of PCR Amplified E3/19K Gene into KT-3B

i. PCR Amplification of E3/19K Gene

The Ad2 DNA E3/19K gene, including the amino terminal signal sequence, followed by the intraluminal domain and carboxy terminal cytoplasmic tail which allow the E3/19K protein to embed itself in the endoplasmic reticulum (ER), is located between viral nucleotides 28,812 and 29,288. Isolation of the Ad2 E3/19K gene from the viral genomic DNA is accomplished by PCR amplification, with the primer pair shown below:

The forward primer corresponds to the Ad2 nucleotide sequences 28,812 to 28,835. (SEQUENCE ID No. 3)

5'-TATATCTCCAGATGAGGTACATGATTTTAGGCTTG-3'

The reverse primer corresponds to the Ad2 nucleotide sequences 29,241 to 29,213. (SEQUENCE ID No. 4)

5'-TATATATCGATTCAAGGCATTTTCTTTTCATCAATAAAAC-3'

In addition to the Ad2 complementary sequences, both primers contain a five nucleotide "buffer sequence" at their 5' ends for efficient enzyme digestion of the PCT amplicon products. This sequence in the forward primer is followed by the Xho I recognition site and by the Cla I recognition site in the reverse primer. Thus, in the 5' to 3' direction, the E3/19K gene is flanked by Xho I and Cla I recognition sites. Amplification of the E3/19K gene from Ad2 DNA is accomplished with the following PCR cycle protocol:

Temperature°C	Time (min)	No. Cycles
94	2.0	1
94	0.5	
55	0.17	5
72	3.5	
94	0.5	30
70	3.5	
72	10.0	10

ii. Ligation of PCR Amplified E3/19K Gene into KT-3B

The E3/19K gene from the SP-E3/19K construct, approximately 780 bp in length, is removed and isolated by 1% agarose/TBE gel electrophoresis. The Xho I-Cla I E3/19K fragment is then ligated into the KT-3B retroviral backbone. This construct is designated KT-3B/E3/19K. It is amplified by transforming *E. coli* , DH5 alpha bacterial strain (Bethesda Research Labs, Gaithersburg, Maryland) with the KT-3B/E3/19K construct. Specifically, the bacteria is transformed with 1-100 ng of ligation reaction mixture DNA. The transformed bacterial cells are plated on LB plates containing ampicillin. The plates are incubated overnight at 37°C, bacterial colonies are selected and DNA prepared from them. The DNA is digested with Xho I and Cla I. The expected endonuclease restriction cleavage fragment sizes for plasmids containing the E3/19K gene are 780 and 1,300 bp.

C. Transduction of Packaging Cell Line DA with the Recombinant Retroviral Vector KT-3B/E3/19K

i. Plasmid DNA Transfection

293 2-3 cells (a cell line derived from 293 cells ATCC No. CRL 1573, (WO 92/05266) 5.0 x 10^5 cells are seeded at approximately 50% confluence on a 6 cm tissue culture dish. The following day, the media is replaced with 4 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA coprecipitation is performed by mixing 10.0 µg of KT-3B/E3/19K plasmid and 10.0 µg MLP G plasmid with a 2M $CaCl_2$ solution, adding a 1x HEPES buffered saline solution, pH 6.9, and incubating for 15 minutes at room temperature. The calcium phosphate-DNA coprecipitate is transferred to the 293 2-3 cells, which are then incubated overnight at 37°C, 5% CO_2. The following morning, the cells are rinsed three times in 1x PBS, pH 7.0. Fresh media is added to the cells, followed by overnight incubation at 37°C, 10% CO_2. The following day, the media is collected off the cells and passed through a 0.45 µ filter. This supernatant is used to transduce packaging and tumor cell lines. Transient vector supernatant for other vectors are generated in a similar fashion.

ii. Packaging Cell Line Transduction

Packaging cell line transduction is performed as described in Example 2C.

iii. Detection Of Replication Competent Retroviruses

Detection of replication competent retroviruses is performed as described in Example 2D

D. Transduction of Cell Lines with the Recombinant Retroviral Vector KT-3B/E3/19K

The following adherent human and murine cell lines are seeded at 5×10^5 cells/10 cm dish with 4 μg/ml polybrene: HT 1080, Hela, and BC10ME. The following day, 1.0 ml of filtered supernatant from the DA E3/19K pool is added to each of the cell culture plates. The following day, 800 μg/ml G418 is added to the media of all cell cultures. The cultures are maintained until selection is complete and sufficient cell numbers are generated to test for gene expression. The transduced cell lines are designated HT 1080-E3/19K, Hela-E3/19K and BC10ME-E3/19K, respectively.

EBV transformed cell lines (BLCL), and other suspension cell lines, are transduced by co-cultivation with irradiated producer cell line, such as DA-E3/19K. Specifically, irradiated (10,000 rads) producer line cells are plated at 5.0×10^5 cells/6 cm dish in growth media containing 4 μg/ml polybrene. After the cells have been allowed to attach for 2-24 hours, 10^6 suspension cells are added. After 2-3 days, the suspension cells are removed, pelleted by centrifugation, resuspended in growth media containing 1mg/ml G418, and seeded in 10 wells of a round bottom 96 well plate. The cultures were expanded to 24 well plates, then to T-25 flasks.

E. Expression of E3/19K in the Recombinant Retroviral Vector Construct KT3B-E3/19K

i. Western Blot Analysis

Radio-immuno precipitation assay (RIPA) lysates are made from selected cultures for analysis of E3/19K expression. RIPA lysates are prepared from confluent plates of cells. Specifically, the media is first aspirated off the cells. Depending upon the size of the culture plate containing the cells, a volume of 100 to 500 ml ice cold RIPA lysis buffer (10 mM Tris, pH 7.4; 1% Nonidet P40; 0.1% SDS; 150 mM NaCl) is added to the cells.

Cells are removed from plates using a micropipet and the mixture is transferred to a microfuge tube. The tube is centrifuged for 5 minutes to precipitate cellular debris and the supernatant is transferred to another tube. The supernatants are electrophoresed on a 10% SDS-polyacrylamide gel and the protein bands are transferred to an Immobilon membrane in CAPS buffer (10 mM CAPS (Aldrich, Milwaukee, WI) pH 11.0; 10% methanol) at 10 to 60 volts for 2 to 18 hours. The membrane is transferred from the CAPS buffer to 5% Blotto (5% nonfat dry milk; 50 mM Tris, pH 7.4; 150 mM NaCl; 0.02% Na azide, and 0.05% Tween 20) and probed with a mouse monoclonal antibody to E3/19K (Severinsson *et al.*, *J. Cell. Biol. 101*:540-547, 1985). Antibody binding to the membrane is detected by the use of ^{125}I-Protein A.

ii FACS Analysis of KT3B-E3/19k-Vector Transduced Cells to Demonstrate Decreased Levels of Class I Expression Compared to Non-Transduced Cells.

Cell lines transduced with the KT3b-E3/19K-vector are examined for MHC class I molecule expression by FACS analysis. Non-transduced cells are also analyzed for MHC class I molecule expression and compared with E3/19K transduced cells to determine the effect of transduction on MHC class I molecule expression.

Murine cell lines, BC10ME, BC10ME-E3/19K, P815 (ATCC No. TIB 64), and P815-E3/19K, are tested for expression of the H-2D^d molecule on the cell surface. Cells grown to subconfluent density are removed from culture dishes by treatment with Versene and washed two times with cold (4°C) PBS plus 1% BSA and 0.02% Na-azide (wash buffer) by centrifugation at 200g. Two million cells are placed in microfuge tubes and pelleted in a microfuge at 200g before removing the supernatant. Cell pellets are resuspended with the H-2D^d-specific Mab 34-2-12s (50ml of a 1:100 dilution of purified antibody, ATCC No. HB 87) and incubated for 30 min at 4°C with occasional mixing. Antibody labeled cells are washed two times with 1 ml of wash buffer (4°C) prior to removing the supernatant. Cells are resuspended with a biotinylated goat anti-mouse kappa light chain Mab (50ml, of a 1:100 dilution of purified antibody) (Amersham, Arlington Height, IL) and incubated for 30 min at 4°C. Cells are washed, resuspended with 50ml of avidin conjugated FITC (Pierce, Rockford, IL), and incubated for 30 min at 4°C. The cells are washed once more, resuspended in 1 ml of wash buffer, and held on ice prior to analysis on a FACStar Analyzer (Becton Dickinson, Los Angeles, CA). The mean fluorescence intensity of transduced cells is compared with that of non-transduced cells to determine the effect E3/19K protein has on surface MHC class I molecule expression.

F. Administration of Vector Construct

i Rat Administration Protocol

Rats are anesthetized and one eye is instilled with 5 to 100 µl of retroviral vector particles at a concentration of 10^5 to 10^{10} cfu/ml in formulation buffer, with or without 4 to 8 µg/ml of polybrene or other transduction enhancing excipient,. Five to one hundred µl of solution containing formulation buffer only is added to the other eye to be used as a control. The solution is allowed to incubate for 1 hour before each eye is rinsed 3 times with 100 µl of saline . Two to seven days following the treatment, the rat is sacrificed, the cornea is removed, and homogenized in 2 ml ice cold RIPA lysis buffer. Expression of E3/19K is detected by Western blot analysis as described in Example 6Ei.

ii Human Administration Protocol

Although the rate of corneal transplant rejection is relatively low, the current therapy for those with rejection requires continuous treatment of steroid compounds. This eventually leads to cataract formation, requiring surgery. Therefore, the introduction of a retroviral vector expressing the E3/19K would prevent the need for such a steroid regimen. Ten to five hundred µl of retroviral vector particles at a concentration of 10^5 to 10^{10} cfu/ml in formulation buffer, with or without 4 to 8 µg/ml of polybrene or other transduction enhancing excipient in formulation buffer, are administered to the eye of a patient lying in a prone position. The solution is allowed to incubate for 15 to 30 minutes before being washed with saline.

Alternatively, the cornea may be incubated for 1 hour in 1 ml of retroviral vector particles at a concentration of 10^5 to 10^{10} cfu/ml in formulation buffer, with or without 4 to 8 µg/ml of polybrene or other transduction enhancing excipient, just prior to surgical attachment. In either of the above cases, the progress of the transplant is monitored by visually observing tissue viability.

EXAMPLE 7

Intranasal Administration of Retroviral Vectors Expressing Factor VIII

A. Construction of Full-Length and B Domain Deleted Factor VIII cDNA Retrovector™

The construction of the full-length and B domain deleted Factor VIII retrovectors are described in Example 2A

B. Administration of Vector Construct

i Rat Administration Protocol

The nasal route has been shown to be effective for the administration of a number of molecules due to the extensive network of capillaries located under the nasal mucosa. This facilitates effective systemic absorption and when the drug is administered with absorption promoters, absorption occurs rapidly with high bioavailability (review in Gizurarson, *et al.*, *Acta Pharm* 2:105, 1990).

One group of six Fischer-344 rats are used for nasal administration of the retroviral vector particles for Factor VIII. One to fifty µl of retroviral vector particles at 10^6 to 10^{11} cfu/ml in formulation buffer, with or without 4 to 8 µg/ml of polybrene or other transduction enhancing excipients are applied with a pipette inserted about 3 to 5 mm into each nostril. Another group is administered formulation buffer without vector in the same manner. Blood samples are collected from the jugular or tail vein 1 to 14 days later and assayed for factor VIII production as described in Example 2Hi.

ii Human Administration Protocol

Several types of drug delivery devices for the nasal cavity exists (reviewed in Chien, Y. *et al.*, *Crit Rev Therap Drug Carr Sys*, 4:67, 1987). These systems include nasal spray, nose drops, saturated cotton pledget, aerosol spray, and insufflator. The meter-dose nebulizer can deliver a predetermined volume of the formulation to the nasal cavity.

Two groups of patients are used in this study. One group of patients receives 100 to 500 µl of retroviral vector particles at 10^6 to 10^{11} cfu/ml in formulation buffer, with or without 4 to 8 µg/ml of polybrene or other transduction enhancing excipients, applied to each nostril via nasal spray or nasal drops. Another group receives formulation buffer only applied in the same manner. Blood samples are collected 1 to 14 days later and assayed for factor VIII production as described in Example 2Bi.

EXAMPLE 8

Preservation of A Recombinant Retrovirus

A. Lactose Formulation of a Recombinant Retrovirus

Crude recombinant retrovirus is obtained from a Celligan bioreactor (New Brunswick, New Brunswick, NJ) containing DA cells transformed with the recombinant retrovirus bound to the beads of the bioreactor matrix. The cells release the recombinant retrovirus into the growth media that is passed over the cells in a continuous flow process. The media exiting the bioreactor is collected and passed initially through a 0.8 micron filter then through a 0.65 micron filter to clarify the crude recombinant retrovirus. The filtrate is concentrated utilizing a cross flow concentrating system (Filtron, Boston, MA). Approximately 50 Units of DNase (Intergen, New York, NY) per ml of concentrate is added to digest exogenous DNA. The digest is diafiltrated using the same cross flow system to 150 mM NaCl, 25 mM tromethamine, pH 7.2. The diafiltrate is loaded onto a Sephadex S-500 gel column (Pharmacia, Piscataway, NJ), equilibrated in 50 mM NaCl, 25 mM tromethamine, pH 7.4. The purified recombinant retrovirus is eluted from the Sephadex S-500 gel column in 50 mM NaCl, 25 mM tromethamine, pH 7.4.

The formulation buffer containing lactose was prepared at a 2X concentrated stock solution. The formulation buffer contains 25 mM tromethamine, 70 mM NaCl, 2 mg/ml arginine, 10 mg/ml HSA, and 100 mg/ml lactose in a final volume of 100 mls at a pH 7.4.

The purified recombinant retrovirus is formulated by adding one part 2X lactose formulation buffer to one part S-500 purified recombinant retrovirus. The formulated recombinant retrovirus can be stored at -70°C to -80°C or dried.

The formulated retrovirus is lyophilized in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer (Edwards High Vacuum, Tonawanda, NY). When the freeze drying cycle is completed, the vials are stoppered under a vacuum following a slight nitrogen gas bleeding. Upon removal, vials are crimped with aluminum seals.

In the given lactose study, formulated liquid product was stored at both -80° C and at -20°C cycling freezer. In Figure 15 viral infectivity of these samples were compared to the viral infectivity of lyophilized samples. The lyophilized samples were stored at -20°C, refrigerator temperature and room temperature. Activity of the samples upon reconstitution are determined by titer assay.

The lyophilized recombinant retrovirus is reconstituted with 1.0 ml water. The infectivity of the reconstituted recombinant retrovirus is determined by a titer activity

assay. The assay is conducted on HT 1080 fibroblasts or 3T3 mouse fibroblast cell line (ATCC No. CCL 163). Specifically, 1 x 10^5 cells are plated onto 6 cm plates and incubated overnight at 37°C, 10% CO_2. Ten microliters of a dilution series of reconstituted recombinant retroviruses are added to the cells in the presence of 4 mg/mL polybrene (Sigma, St. Louis, MO) and incubated overnight at 37°C, 10% CO_2. Following incubation, cells are selected for neomycin resistance in G418 containing media and incubated for 5 days at 37°C, 10% CO_2. Following initial selection, the cells are re-fed with fresh media containing G418 and incubated for 5-6 days. After final selection, the cells are stained with Commassie blue for colony detection. The titer of the sample is determined from the number of colonies, the dilution, and the volume used.

Figure 15 demonstrates that storage in lyophilized form at -20°C to refrigerator temperatures retains similar viral activity as a recombinant retrovirus stored in liquid at -80 to -20°C permitting less stringent temperature control during storage.

B. Mannitol Formulation of a Recombinant Retrovirus

The recombinant retrovirus utilized in this example was purified as described in Example 8A.

The formulation buffer containing mannitol was prepared as a 2X concentrated stock solution. The formulation buffer contains 25 mM tromethamine, 35 mM NaCl, 2 mg/ml arginine, 10 mg/ml HSA and 80 mg/ml mannitol at a final volume of 100 mls at a pH 7.4.

The purified recombinant retrovirus is formulated by adding one part mannitol formulation buffer to one part S-500 purified recombinant retrovirus. The formulated recombinant retrovirus can be stored at this stage at -70°C to -80°C or dried.

The formulated retrovirus is dried in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer. When the freeze drying cycle is completed, the vials are stoppered under a vacuum following nitrogen gas bleeding to 700 mbar. Upon removal, vials are crimped with aluminum seals.

In the given mannitol study, formulated liquid product was stored at both -80 °C and at -20°C in cycling freezers. The viral infectivity of these samples were compared to the viral infectivity of lyophilized samples, Figure 16 The lyophilized samples were stored at -20°C, refrigerator temperature and room temperature. Activity of the samples upon reconstitution are determined using the titer assay described in Example 8A.

Figure 16 demonstrates that storage in lyophilized form at -20°C to refrigerator temperature retains significant viral activity as compared to recombinant retrovirus stored in liquid at -80°C or -20°C, permitting less stringent temperature control during storage.

C. Trehalose Formulation of a Recombinant Retrovirus

The recombinant retrovirus utilized in this example was purified as described in Example 8A.

The formulation buffer containing trehalose was prepared as a 2X concentrated stock solution. The formulation buffer contains 25 mM tromethamine, 70 mM NaCl, 2.0 mg/ml arginine, 10.0 mg/ml HSA and 100 mg/ml trehalose at a final volume of 100 mls at a pH 7.2.

The purified recombinant retrovirus is formulated by adding one part trehalose formulation buffer to one part S-500 purified recombinant retrovirus. The formulated recombinant retrovirus can be stored at this stage at -70°C to -80°C or dried.

The formulated retrovirus is dried in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer. When the freeze drying cycle is completed, the vials are stoppered under a vacuum following nitrogen gas bleeding to 700 mbar. Upon removal, vials are crimped with aluminum seals.

In the given trehalose study, formulated liquid product was stored at both -80 °C and at -20°C in cycling freezers. The viral infectivity of these samples was compared to the viral infectivity of lyophilized samples, Figure 17. The lyophilized samples were stored at -20°C, refrigerator temperature and room temperature. Activity of the samples upon reconstitution are determined using the titer assay as described in Example 8A.

Figure 17 demonstrates that storage in lyophilized form at -20°C to refrigerator temperature retains similar viral activity as compared to recombinant retrovirus stored in liquid at -80°C to -20°C permitting less stringent temperature control during storage.

Viral infectivity of liquid formulated recombinant retrovirus samples stored at -80°C was compared to viral infectivity of lyophilized formulated recombinant retrovirus stored at -20°C. Initially, a bulk of recombinant retrovirus was received and formulated in four different ways as shown below. The formulated recombinant retrovirus was then frozen in bulk for 1.5 months subsequent to being quick thawed and freeze dried. Positive controls were stored at -80°C for comparison with lyophilized samples which were stored at -20°C after freeze-drying. The formulations are listed below:

Formulation	Sugar Concentration (mg/ml)	Buffer Concentration (mM tromethamine)	Salt Concentration (mM NaCl)	Arginine Concentration (mg/ml)	Human Serum Albumin Concentration (mg/ml)

Mannitol	40	25	25	1	5
Lactose	40	25	75	1	5
Sucrose	50	25	60	1	5
Trehalose	50	25	60	1	5

In the graphs of Figure 18, the y-axis on each of the 4 graphs (A, B, C, D) represent the normalized titer. At an initial time point after lyophilization, t = 0, a titer value was established for both the -80°C liquid sample and the -20°C lyophilized sample. At each time point of the stability study, the titer obtained was divided by the zero time point titer value and the % of original entered onto the graph.

The data demonstrates that post-lyphilization activity is maintained in the lyophilized sample (stored at -20°C) relative to the liquid sample (stored at -80°C). The formulated lyophilized recombinant retrovirus was stored in a -20°C freezer (a frost-free cycling freezer). Comparison to the formulated liquid recombinant retrovirus stored at -80° C indicates the lyophilized form permits less stringent control of storage conditions.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:CHIORN VIAGENE, INC.

(ii) TITLE OF INVENTION: NON-TRAUMATIC ADMINISTRATION OF GENE DELIVERY VEHICLES

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(A) APPLICATION NUMBER: PCT - Unassigned
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(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAGAGATGGG GGAGGCTAAC TGAG 24

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATCCTCAGT TAGCCTCCCC CATCTCTC 28

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TATATCTCCA GATGAGGTAC ATGATTTTAG GCTTG 35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TATATATCGA TTCAAGGCAT TTTCTTTTCA TCAATAAAAC 40

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 77 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGTGAATTCG AGCTCGGTAC CCGGGGATCC TCTAGAGTCG ACCTGCAGGC ATGCAAGCTT 60

GGCGTAATCA TGGTCAT 77

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ala Arg Glu Met Gly Glu Ala Asn 1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCCGAGAGAT GGGGGAGGCT AACTGAG 27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGCTCTCTA CCCCCTCCGA TTGACACCTA G 31

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Thr Ile Met Thr Met 1 5

Claims

1. A method of introducing a nucleic acid molecule to an animal comprising non-traumaticly administering a composition comprising a gene delivery vehicle to the animal, said gene delivery vehicle directing the expression of at least one substance in a host cell containing said gene delivery vehicle, the substance not naturally expressed by said gene delivery vehicle, in combination with a pharmaceutically acceptable carrier or diluent.

2. A method of introducing a nucleic acid molecule to an animal comprising non-traumaticly administering a composition comprising a gene delivery vehicle to the animal, said gene delivery vehicle containing at least one biologically active nucleic acid sequence wherein such biological activity is not naturally contained within said gene delivery vehicle, in combination with a pharmaceutically acceptable carrier or diluent.

3. A method of introducing a nucleic acid molecule to an animal comprising non-traumaticly administering a composition comprising a gene delivery vehicle to the animal, said gene delivery vehicle containing a nucleic acid sequence that is not naturally contained within said gene delivery vehicle, in combination with a pharmaceutically acceptable carrier or diluent.

4. The method of any one of claims 1-3 wherein said pharmaceutically acceptable carrier or diluent enhances the administration of said gene delivery vehicle.

5. The method of any one of claims 1 or 3 wherein said substance or said biological activity is not exhibited in said host cell prior to said administration.

6. The method of any one of claims 2 or 3 wherein said biological activity is not exhibited within said animal prior to administration.

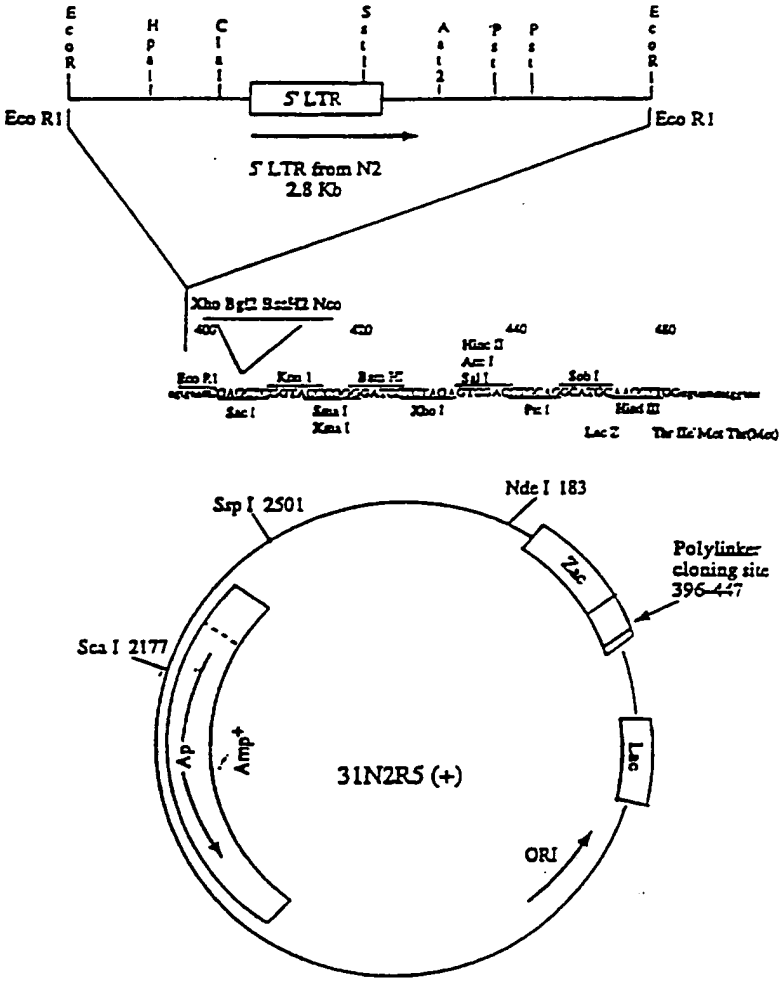
7. The method of any one of claims 2 or 3 wherein said biological activity complements a biological activity present in a host cell within said animal prior to administration.

8. The method of any one of claims 2 or 3 wherein said biological activity activates a biological activity that had not been exhibited in a host cell within said animal prior to administration.

9. The method of any one of claims 2 or 3 wherein said biological activity replaces a biological activity exhibited in a host cell within said animal prior to administration.

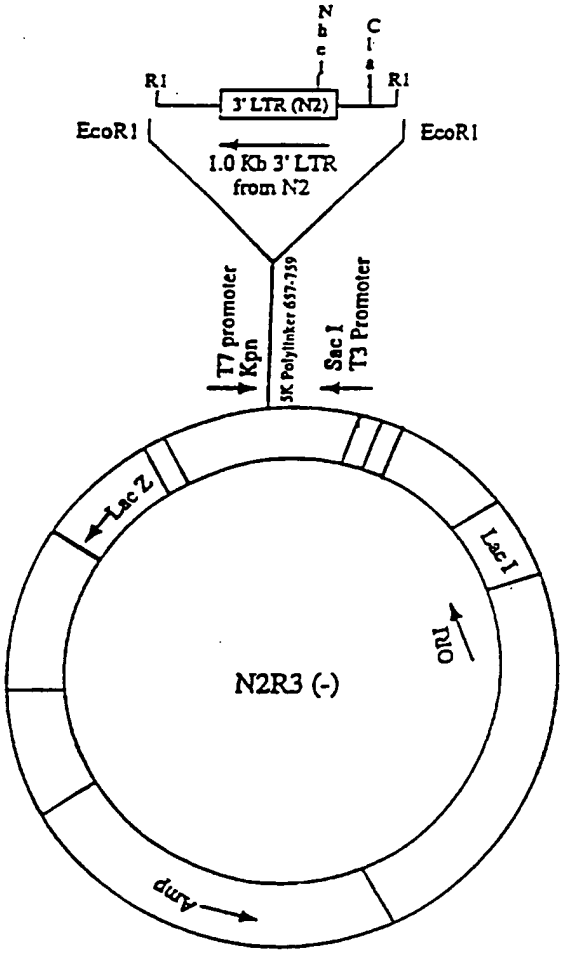
10. The method of any one of claims 2 or 3 wherein said biological activity suppresses a biological activity exhibited in a host cell within said animal prior to administration.

FIGURE 1



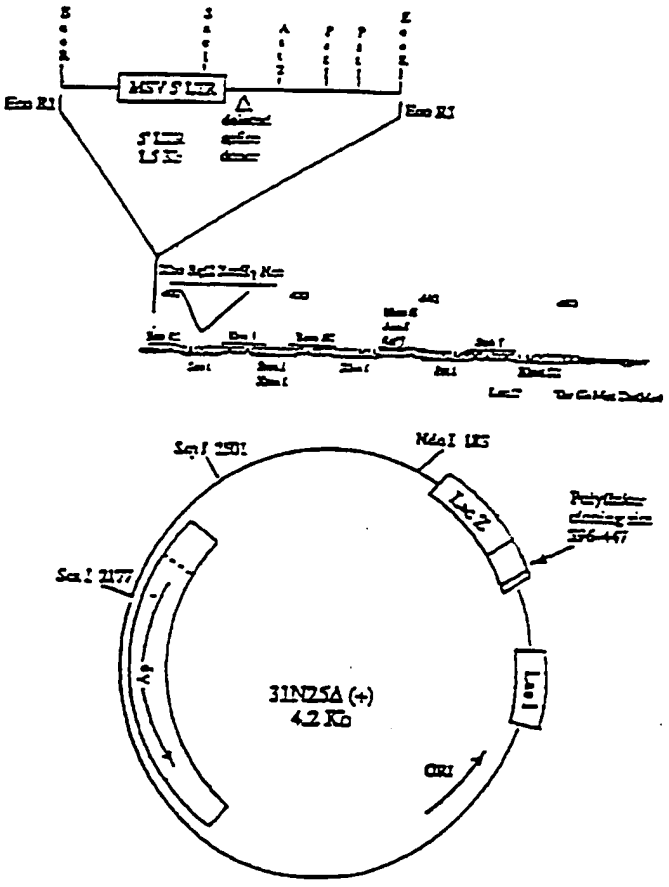
Construction of Vector p31N2R5(+)

FIGURE 2



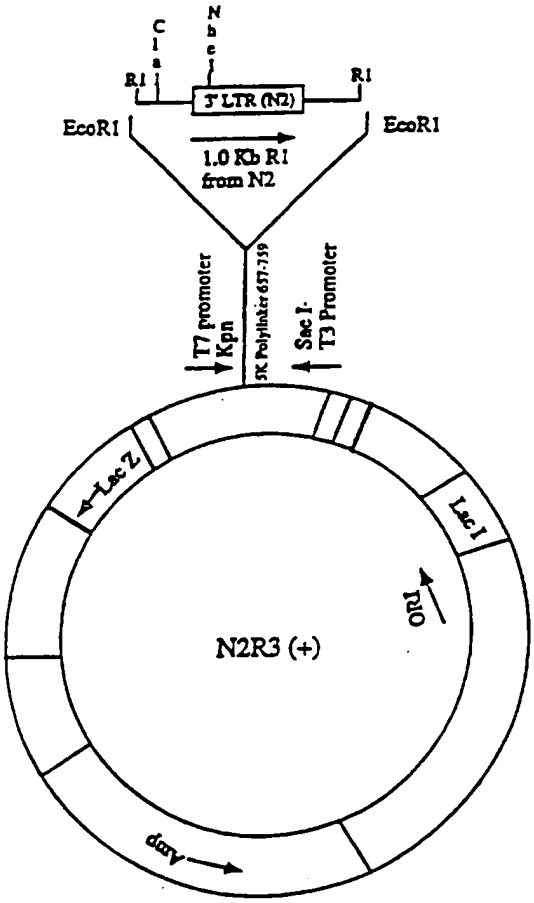
Construction of Vector pN2R3(-)

FIGURE 3



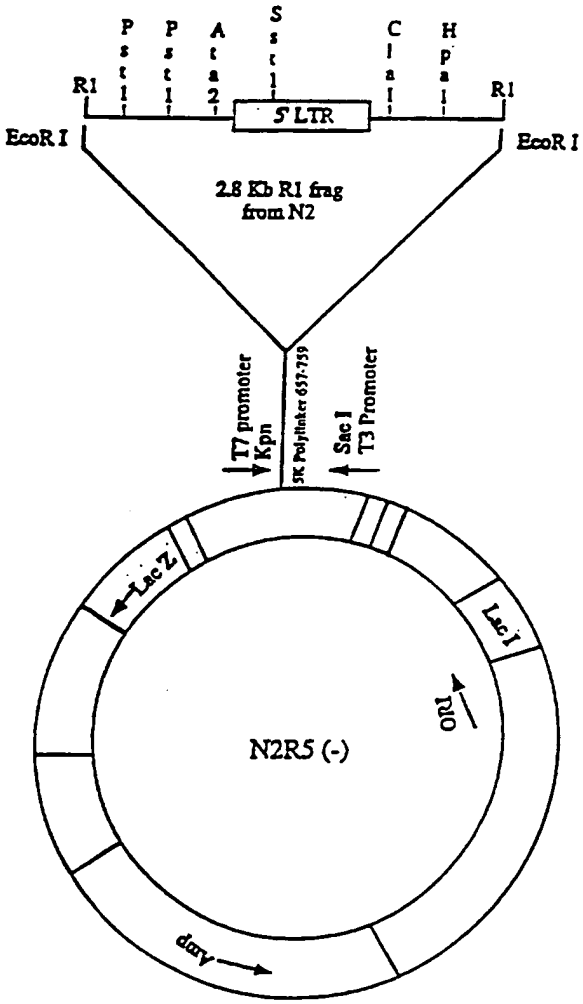
Construction of Vector p3IN2SA(+)

FIGURE 4



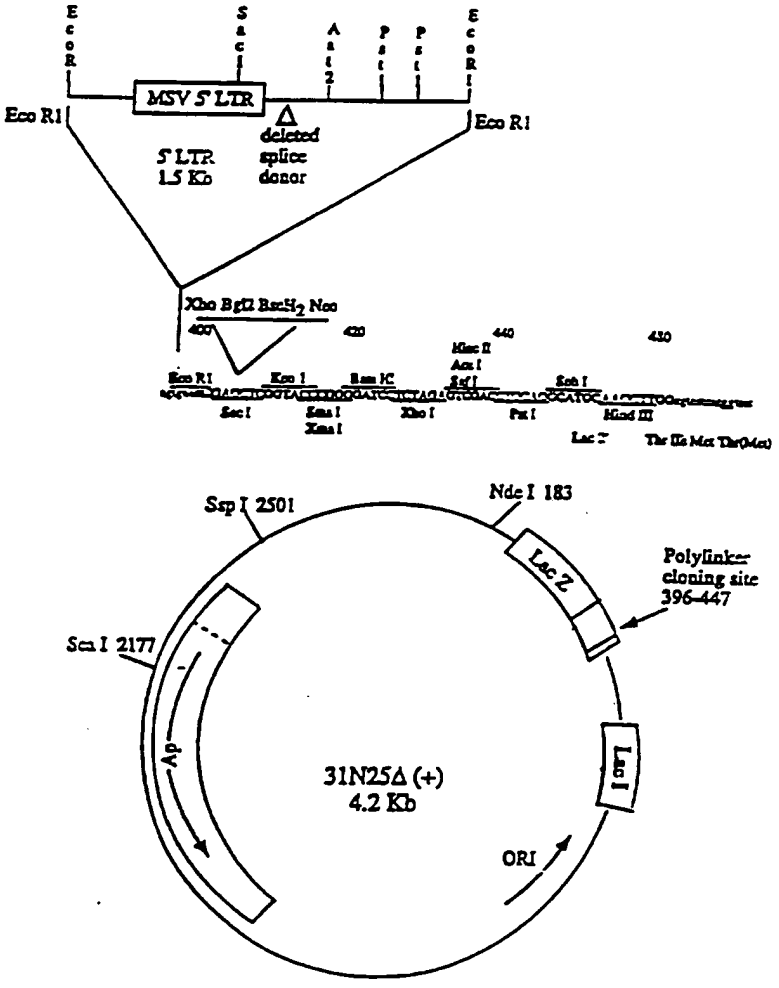
Construction of Vector pN2R3(+)

FIGURE 5



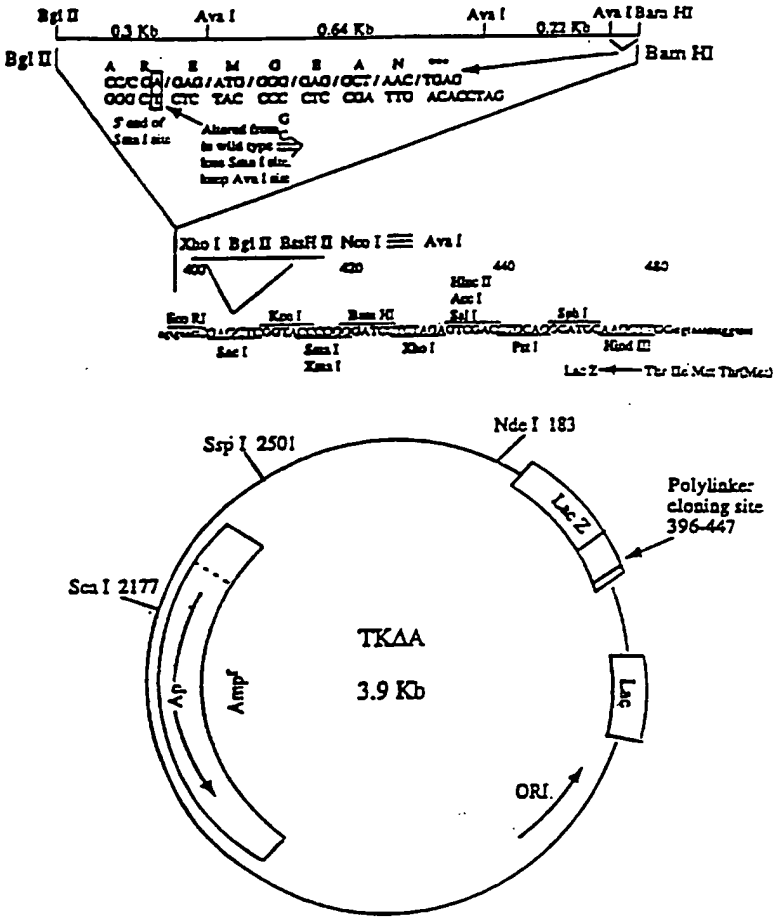
Construction of Vector pN2R5(-)

FIGURE 6



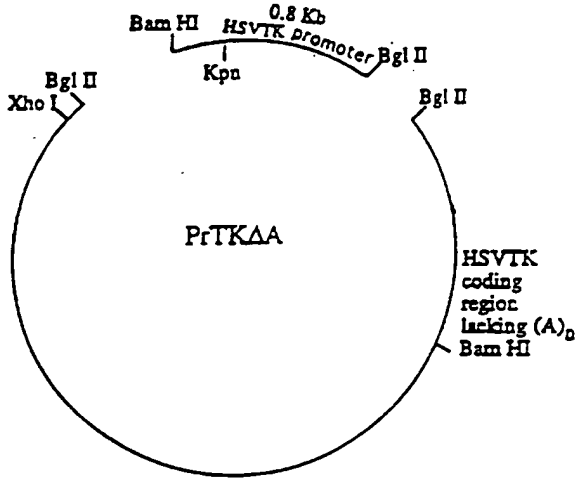
Construction of Vector p31N25Δ(+)

FIGURE 7



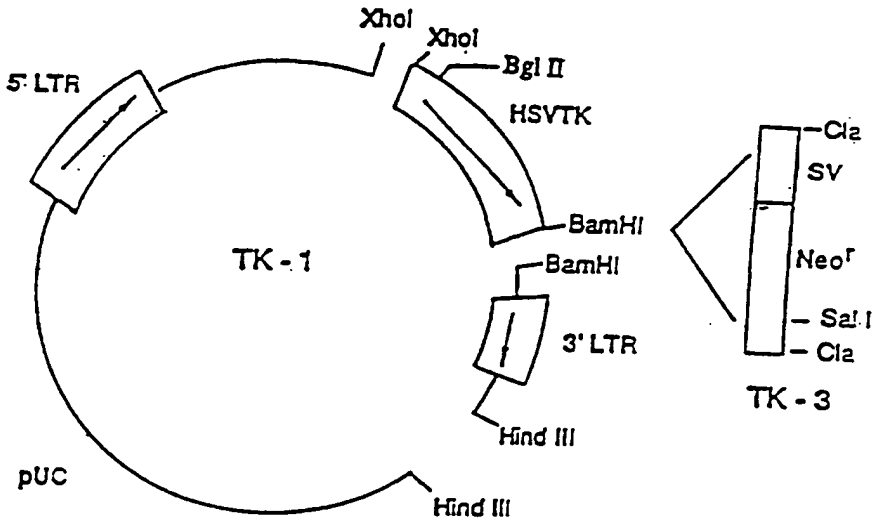
Construction of Vector pTKΔA

FIGURE 8



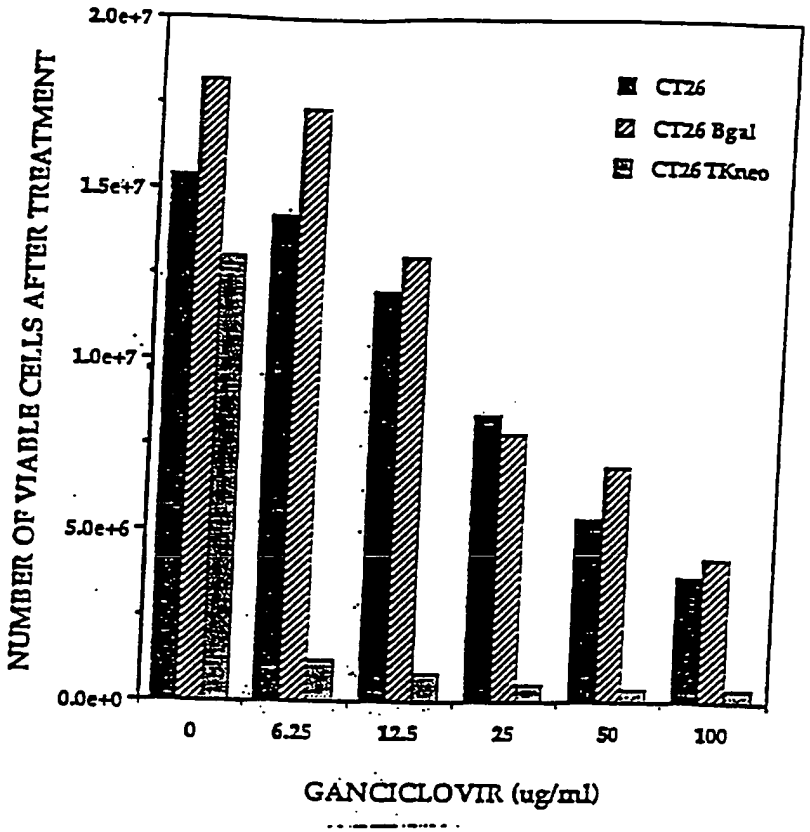
Construction of Vector pPrTKΔA

FIGURE 9



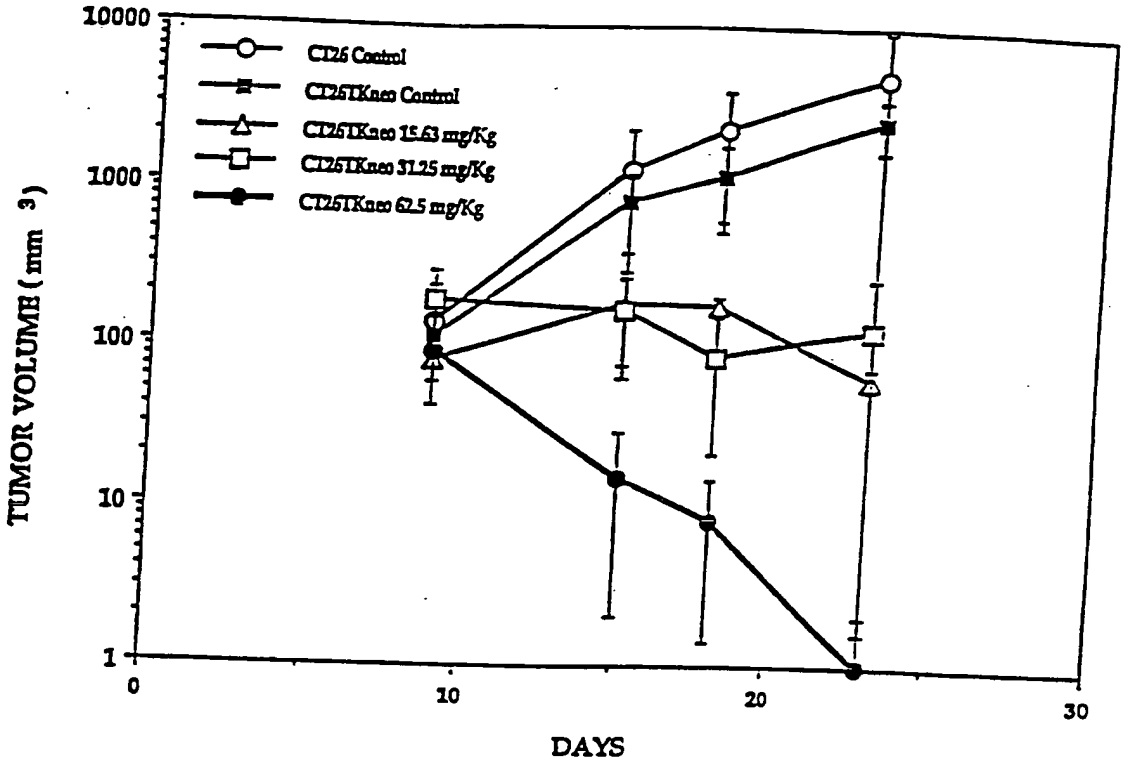
Construction of Retroviral Vectors pTK-1 and pTK-3

FIGURE 10



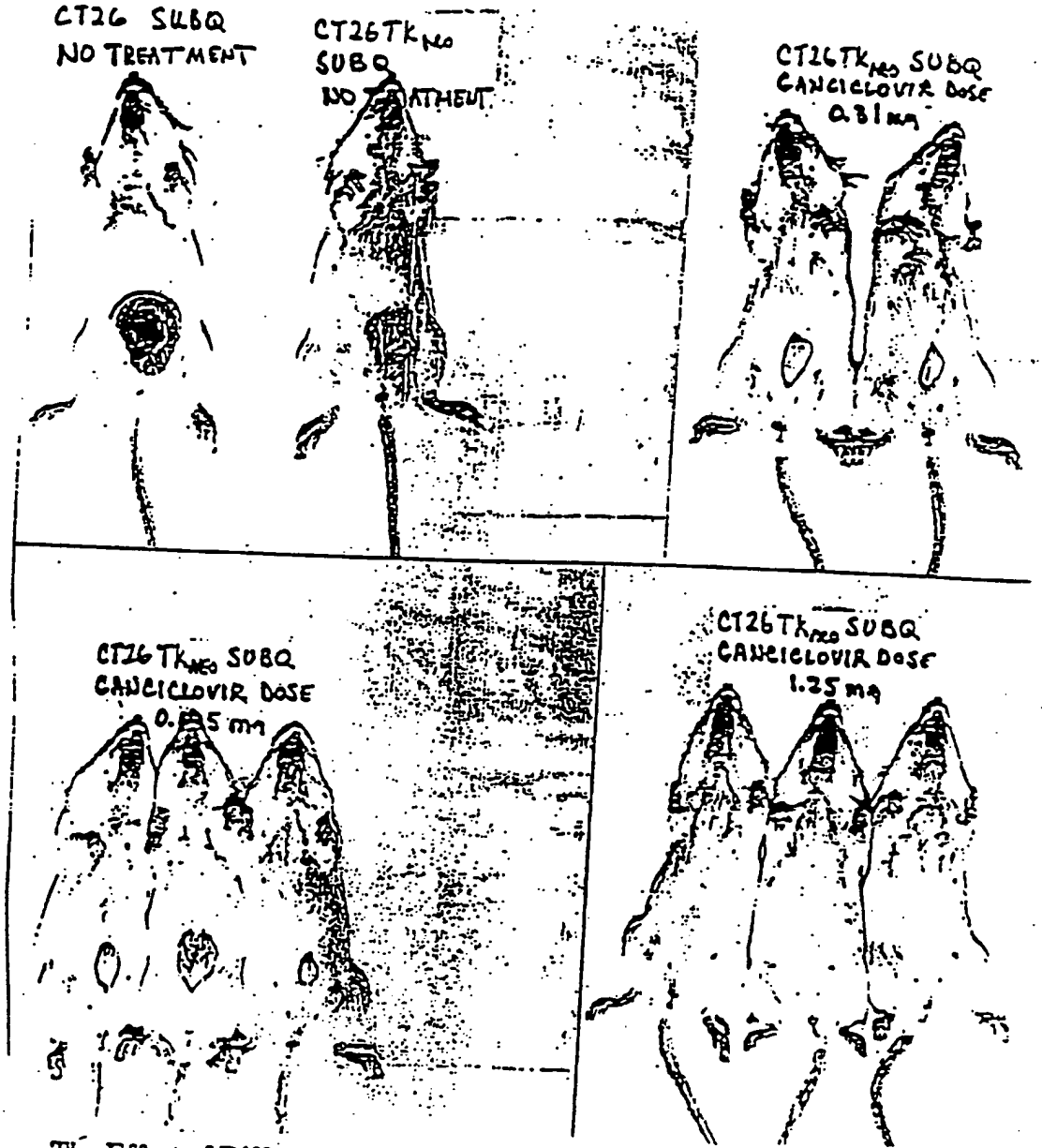
Effect of Ganciclovir on CT26, CT26 βgal and CT26TKNeo Cells

FIGURE 11



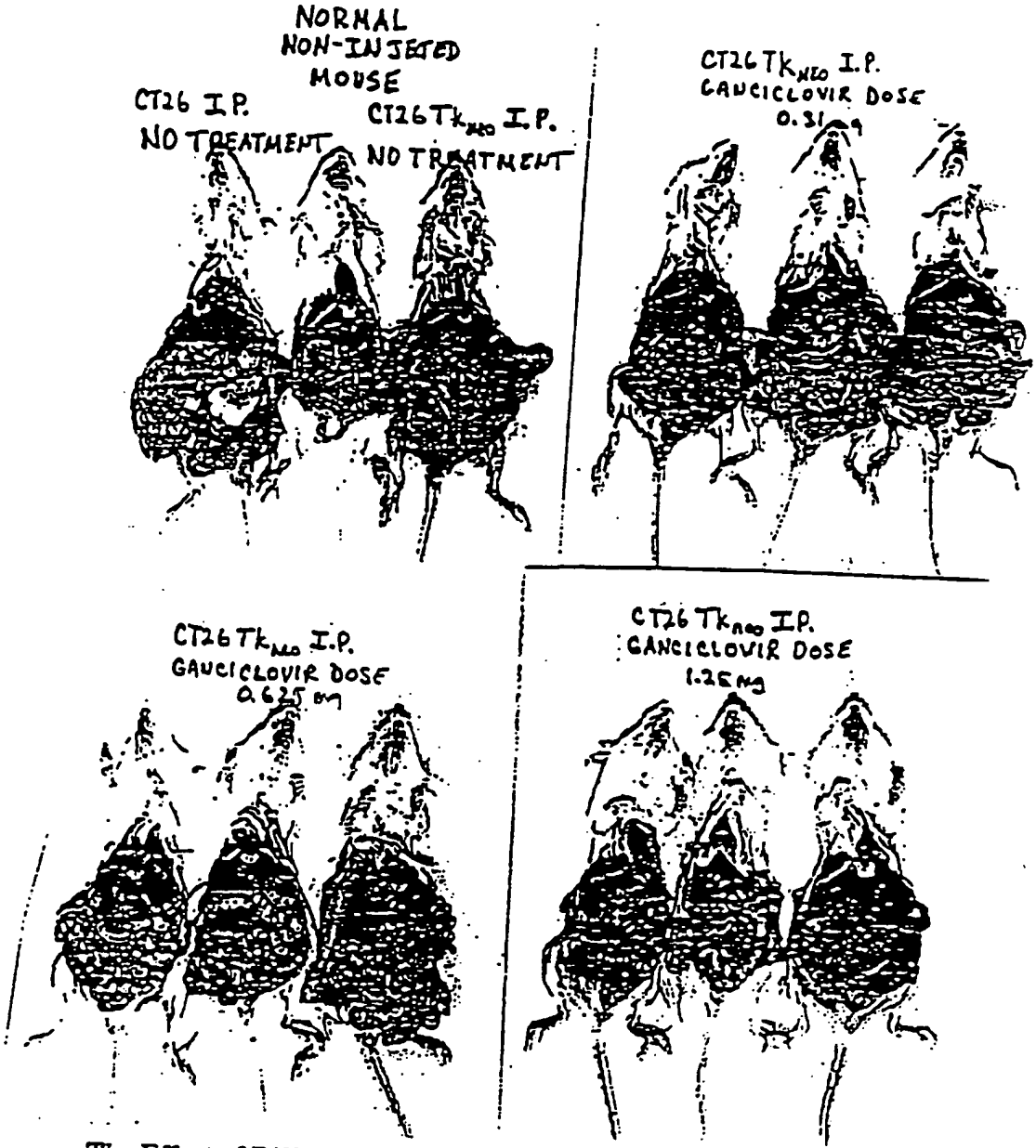
Ganciclovir Dose Study in Mice Injected with CT26 TKNeo

FIGURE 12



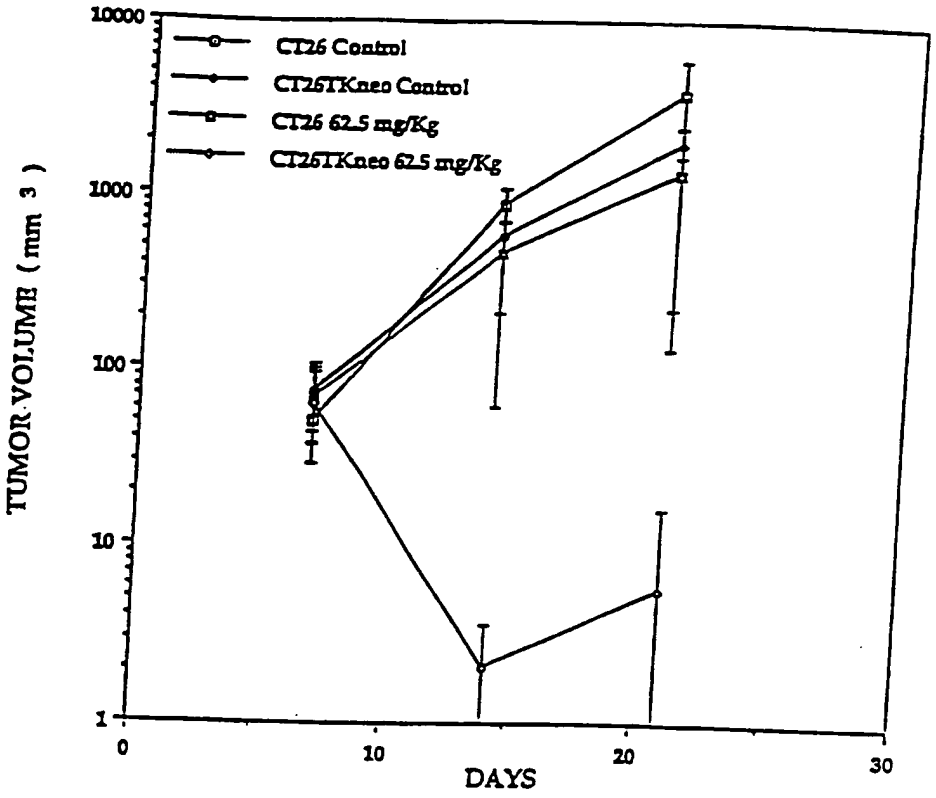
The Effect of Different Dose Regimen of Ganciclovir on Intraperitoneal CT26TKNeo Tumor Growth

FIGURE 13



The Effect of Different Dose Regimen of Ganciclovir on Subcutaneous CT26TKNeo Tumor Growth

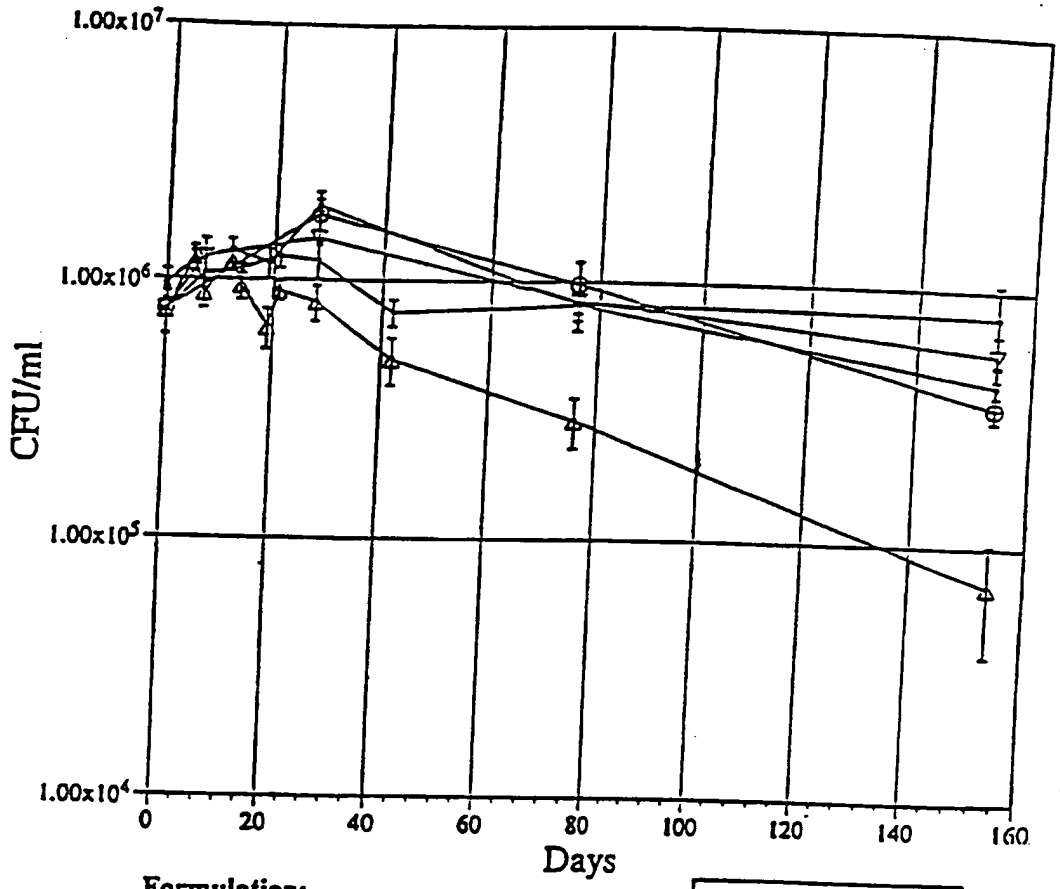
FIGURE 14



Effect of Ganciclovir on the *In Vivo* Growth of CT26 vs. CT26 TKNeo

FIGURE 15

Lactose Stability Study

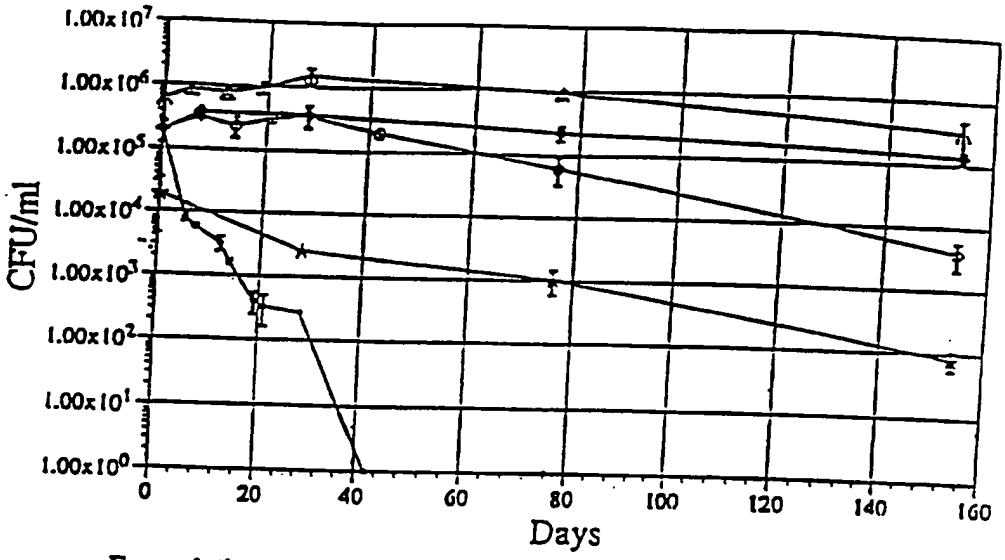


Formulation:

25mM Tris pH 7.2
60mM NaCl
1 mg/ml Arginine
5 mg/ml HSA
50 mg/ml Lactose

FIGURE 16

Mannitol Stability Study



Formulation:

25mM Tris pH 7.2
25mM NaCl
1 mg/ml Arginine
5 mg/ml HSA
40 mg/ml Mannitol

-80°C Liquid
-20°C Liquid
-20°C Lyoph
Refrig. Lyoph
R.T. Lyoph

FIGURE 17

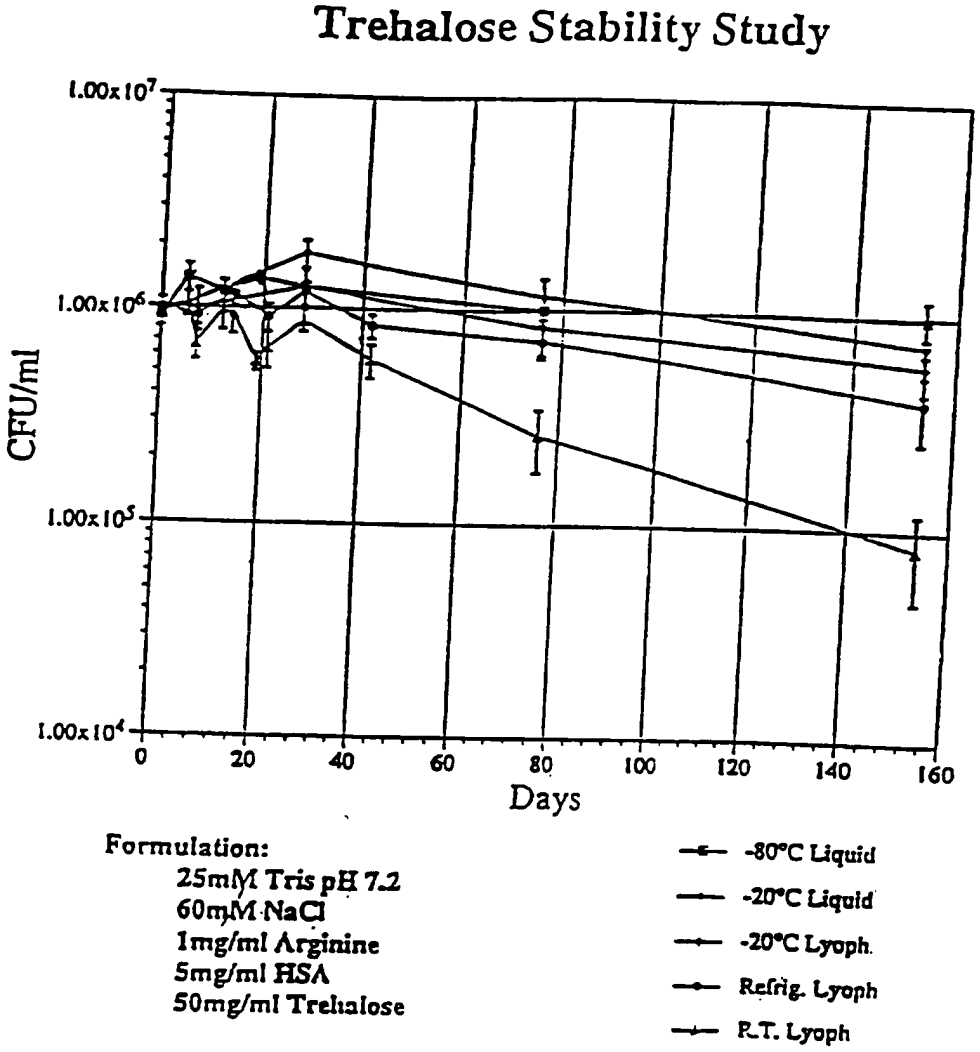
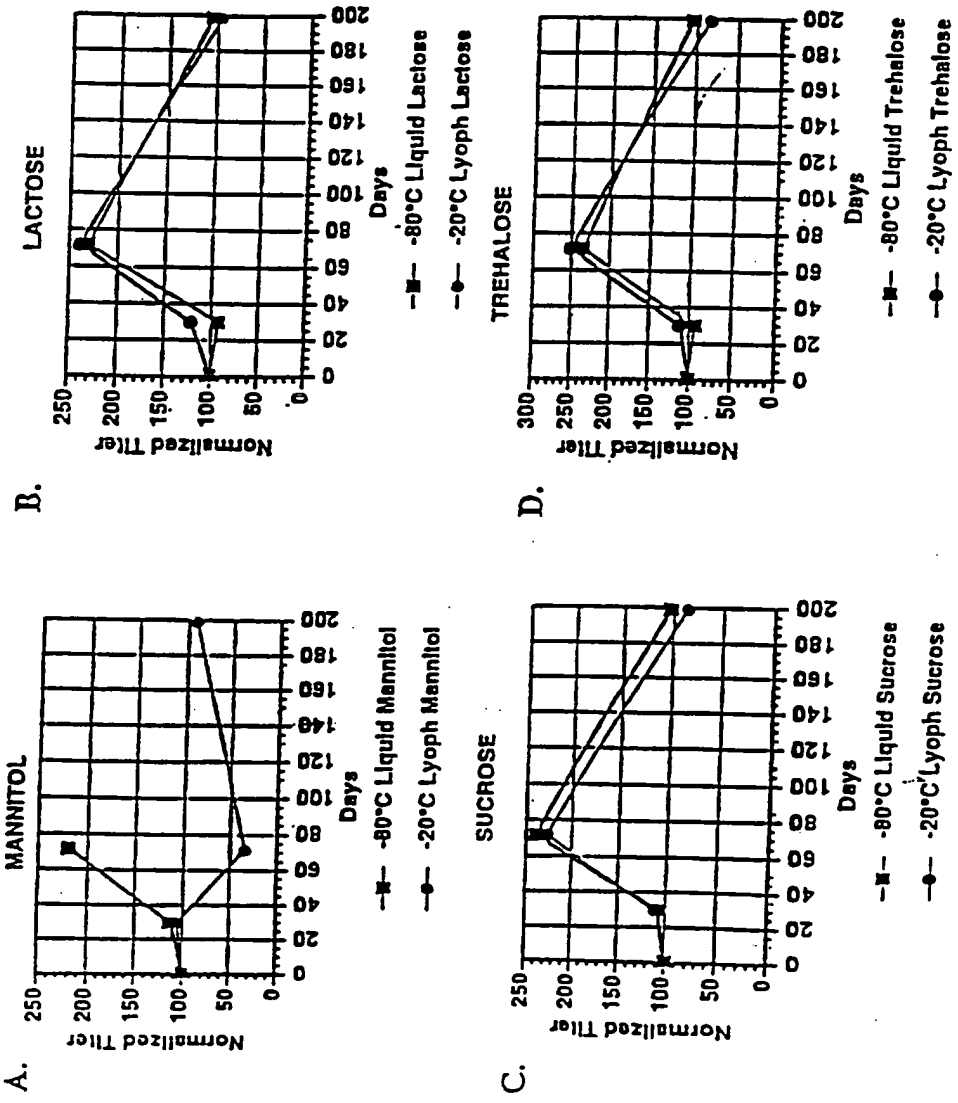

Trehalose Stability Study
1.00x10^7
1.00x10^6
1.00x10^5
1.00x10^4
CFU/ml
0
20
40
60
80
100
120
140
160
Days
Formulation:
25mM Tris pH 7.2
60mM NaCl
1mg/ml Arginine
5mg/ml HSA
50mg/ml Trehalose
-80°C Liquid
-20°C Liquid
-20°C Lyoph.
Refrig. Lyoph
R.T. Lyoph

FIGURE 18


A.
MANNITOL
Normalized Titer
Days
-80°C Liquid Mannitol
-20°C Lyoph Mannitol
B.
LACTOSE
Normalized Titer
Days
-80°C Liquid Lactose
-20°C Lyoph Lactose
C.
SUCROSE
Normalized Titer
Days
-80°C Liquid Sucrose
-20°C Lyoph Sucrose
D.
TREHALOSE
Normalized Titer
Days
-80°C Liquid Trehalose
-20°C Lyoph Trehalose

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